A STUDY OF SOME FACTORS INFLUENCING INTESTINAL FUNCTION IN CALVES.

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CONTENTS

DECLARATION

In accordance with regulation 2.4.15., I hereby declare that this thesis has been composed by myself and consists of my own work.

As required by regulation 2.4.11., it is recorded that some material included in this thesis has been published. Reprints of these papers are contained in Appendix 4.

Signed,

R.J.BYWATER.

SUMMARY

Culture filtrates prepared from a strain of *Escherichia coli* associated with calf diarrhoea were shown to produce fluid accumulation in ligated segments of calf intestine. No activity could be demonstrated *in vitro* in the other experimental systems in which these culture filtrates were tested. The material was therefore examined using Thiry-Vella loops prepared in calves.

Thiry-Vella loops were found to be a useful test preparation for the study of enterotoxin activity, and were used to examine the effect of enterotoxic extracts on net fluid, electrolyte and glucose absorption. It was found that a significant shift towards secretion of fluid, sodium, bicarbonate and chloride occurred in each case in which they were examined. Potassium was not consistently affected, and glucose absorption was unchanged.

The effects of enterotoxin on unidirectional fluxes of water and sodium were studied using isotopic labels. It was found that the net effect on water movement resulted from an increase in exsorption. However, the net effect on sodium movement was mainly the result of decreased insorption. It was suggested that this indicated a complex mechanism for enterotoxin activity.

Culture filtrates prepared from strains of *E.coli* isolated from diarrhoeic but non-septicaemic calves provoked a greater volume of fluid exudate than did filtrates from strains in septicaemic or healthy animals. It was suggested that enterotoxin production may be a graded phenomenon, and moreover, may be commonly encountered in organisms associated with diarrhoea in calves.

Using Thiry-Vella loops as a test preparation, it was shown that enterotoxic activity was distinct from bacterial endotoxin, and that the activity was dialysable using Visking tubing. Ultrafiltration showed that most of the activity was contained in the 1000 to 10,000 molecular weight fraction. The material was stable when heated at 100°C, but showed partial loss of activity when heated at 121°C for two hours.

Evidence of systemic absorption of enterotoxin was obtained in one animal, but could not be confirmed in another. Attempts to inhibit enterotoxin activity with drugs were not successful.

Mucosal lactase was assayed in material from healthy calves and from calves which had died after scouring. Lactase activity was shown to be depressed in the latter group. Normal calves were shown to be able to digest at least twice the normal lactose intake before changes were seen in faeces weight and dry matter, but despite this, lactose tolerance tests showed that there was a significant regression of peak blood glucose rise on faecal dry-matter content. It was therefore suggested that scouring calves may be unable to fully utilise dietary lactose. No effect of enterotoxin on lactase activity could be demonstrated.

GENERAL INTRODUCTION

1. The importance of neonatal diarrhoea in calves.

Diarrhoea in calves ('scour', 'scours' or 'white-scour') was recognised as a problem by Tolnay (1799) and since this time, attempts have been made to assess the incidence and importance of the condition. Many of the surveys which have been carried out have involved relatively small populations of animals at risk thereby limiting the value of the findings, especially in view of the considerable variation which can occur depending on such factors as the standard of management and the season of the year (Lovell 1955; Leech, Macrea & Menzies 1968).

Studies involving a population of more than 3000 calves include those of Lovell and Hill (1940); Withers (1952-53); Sellers, Smith and Wood (1968) and Leech et al, (1968). Figures from these reports show some variation. Withers (1952-53) found an overall mortality of 8.3% but noted considerable variation between regions; losses appeared particularly high in Scotland, and low in beef and dual-purpose herds. Sellers et al, (1968) found an overall mortality of 4.9%, while the most comprehensive survey so far, Leech et al, 1968, showed a mortality of 5-6% during the first year of life.

This latter survey (Leech et al, 1968) which was based on a sample population of 40,173 calves in Great Britain during one year (1962-63) estimated that the total loss in Britain among home-bred calves during the first month of life was about 89,000, while a further 19,000 bought-in calves died within a month of purchase. This survey also showed that gastro-intestinal disorders were responsible for nearly half of the deaths

(44.5%) during the early weeks of life. Other surveys have emphasized that the greatest losses due to diarrhoea occur during the first three weeks of life (Sellers et al, 1968).

It is possible therefore, that 41,000 calves per year may be lost as a result of diarrhoea during the early weeks of life. The value of these animals probably exceeds £500,000 while a further loss results from morbidity, which for 'scouring' was put at 8.9% (Leech et al, 1968). Although many affected animals survive, the recovered animals are frequently unthrifty (BVA, Diseases of Farm Livestock 1957.) The resulting economic loss, together with the cost of treatment, is likely to be considerable.

2. The association between Escherichia coli and gastro-enteric disease in calves.

Jensen (1893) recognised a possible connection between what is now known as Escherichia coli (E.coli) and diarrhoea in calves. Since this time a number of authors have stressed the association between E.coli and gastro-intestinal disease (Smith and Little, 1922; Smith and Orcutt, 1925; Lovell, 1937) so that the complex syndrome is often referred to as 'colibacillosis' (Blood & Henderson, 1968).

Two main types of 'colibacillosis' have been described, the septicaemic and the non-septicaemic type.

(a) The Septicaemic form.

This type of disease is encountered frequently in calves which have been deprived of colostrum (Smith and Orcutt, 1925; Smith, 1962). It is characterised by rapid collapse associated with E.coli bacteraemia. Diarrhoea is of little importance and may be absent (Smith, 1962). Fey & Margadant (1962) showed that 96% of calves dying of septicaemia were agammaglobulinaemic and that

11% of normal calves had low levels of gammaglobulin despite having received colostrum. It is not clear whether the last group received too little colostrum, received it too late, or whether there was a true failure to absorb the macromolecular antibodes during the immediately post-natal period.

It has been found that a comparatively small number of serotypes of E.coli are responsible for deaths due to coliseptacaemia (Sojka, 1965; Gay, 1965) and of these, the strain 078 K80(P) is the most commonly encountered on post-mortem. The link between certain strains of E.coli and this form of the disease seems, therefore, quite clear.

(b) The Non-septicaemic or enteric form.

This form of the disease is seen mainly among calves that have received colostrum, and the bacteria are confined to the intestine. It has been suggested (Gay, 1965) that in some cases absorption of bacterial endotoxin may occur, and that this may be responsible for the rapid death seen in these 'enteric-toxaemic' cases. In the typical enteric form, however, diarrhoea is always seen, and death may or may not occur, depending on the severity. Septicaemia may occur in the later stages of the condition.

The association of enteric scour with abnormal proliferation of E.coli in the upper small intestine was for long accepted as a result of the work of Carpenter & Woods (1924) and Smith and Orcutt (1925) who found large numbers of E.coli in the upper intestine of scouring calves. Doubt was cast on this by the work of Smith (1962) who showed that E.coli was present in the upper small intestine even in normal calves, and moreover, could find no evidence of a proliferation of E.coli in the intestine of scouring calves. He also noted the diversity of phage types in the gut of scouring calves, the absence of incriminating serological findings, and the negative transmission experiments.

He concluded that, with the exception of one field case, he could find no evidence that E.coli was a primary causative factor in the cases he examined. This did not exclude the possibility that E.coli was a secondary factor in the disease.

Gay (1965) in a review of the literature concerning E.coli and neonatal disease in calves, accepted the superior techniques used by Smith (1962) in comparison with the earlier workers. He suggested, however, that the condition described by Smith was probably not true colibacillosis, while that described by Smith & Orcutt (1925) probably was. It is also noteworthy that the calves studied by Smith (1962) were of the Jersey breed, which is particularly vulnerable to disease in early life(Leech et al, 1968) and so the syndrome in this breed may not be typical of others.

The problem remained in this rather ambiguous position until partially clarified by the work of Smith and Halls (1967a) who used ligated loops of intestine to assess the enteropathogenicity of strains of E.coli, and to show furthermore (Smith and Halls, 1967b) that enteropathogenic strains produced an enterotoxin. However, they suggested that these enteropathogenic strains were responsible for only a small proportion of outbreaks of disease.

Viruses have been implicated in diarrhoea in calves. As early as 1943 (Baker) a virus was isolated from calves with enteritis and pneumonia which, after passage through mice, reproduced the syndrome in a calf. There have been more recent reports of virus implication in diarrhoea in calves (Romvari, 1965; Christov, Karadjov, Pavlov & Andreev, 1965; Lambert & Fernelius, 1968). The relevance of viruses in the aetiology of diarrhoea of calves is difficult to assess, but they must remain as a possible factor, either alone or in combination with E.coli.

Diarrhoea in calves can also result from infection with Salmonella spp and Clostridium welchii (Blood & Henderson, 1968). These diseases can usually be diagnosed on the basis of clinical and bacteriological examination, and are seldom confused with typical cases of scour where no specific pathogen can be implicated.

3. The Pathogenesis of diarrhoea in young domestic animals.

There is only a limited understanding of the pathogenesis of diarrhoea in calves (Kohler, 1968) piglets (Kohler, 1968; Thomlinson, 1969) or man (Fordtran, 1967).

The common feature of diarrhoea in the various species is the severe loss of fluid and electrolytes in the faeces. This is a feature of the 'enteric' form of scour in calves (Blaxter & Wood, 1953; Fayet, 1968). The cause of the excessive fluid loss is not understood, even in cases where an enterotoxin-producing organism is present, since the nature and mode of action of the enterotoxin/s has not been defined. Smith and Halls (1967a) reported that of 127 epidemiologically unrelated cases of diarrhoea in calves, only 7 were associated with organisms which produced enterotoxin (i.e. caused dilatation in ligated loops of calf intestine). The possibility must remain that the ligated loop test may not be sufficiently sensitive to detect other than particularly potent enterotoxin producers.

However, Smith and Halls (1967a) suggested that the 120 cases were examples of diarrhoea which were non-infective in origin in the same way as those described earlier (Smith, 1962). At that time he suggested that an 'enzyme-deficiency' may be involved, but did not discount the possibility that bacteria may play a secondary role in the condition. Blaxter & Wood (1953) also suggested that a digestive dysfunction was occurring in the upper small

intestine, so allowing undigested food material to reach the lower gut.

The relationship between the bacterial flora and the digestive and absorptive function of the intestinal mucosa may have a bearing on these cases of diarrhoea.

The intestinal mucosal cells of greatest importance in digestion and absorption are situated in the upper third of the intestinal villus. These cells originate at the base of the crypts, and migrate to the tips of the villus. During the process of migration, maturation of the cells occurs, with development of the enzyme systems associated with the brush-border region (Padykula, 1962; Dahlqvist, 1967). It has been shown that the rate of cell migration in experimental animals can be affected by a number of factors, of which enteritis is one (Abrams, Schneider, Formal and Sprinz, 1963). Moreover, it has been shown that when germ-free mice are placed in a contaminated environment, the mucosal cell turnover rate doubled in eight days (Khoury, Floch & Hersh, 1969). It has been suggested that the increase in migration rate may reach the point where cells are reaching the upper third of the villus (the main site of digestion and absorption) in an immature state (Radostits, 1965; Dowling, 1970). Cells of this type might be expected to have a cuboidal appearance (the shape in which they originated in the crypt) rather than the normal columnar form. Such cuboidal cells have been described by Kenworthy (1970) in germ-free piglets contaminated by a pathogenic strain of E. coli and also by Radostits (1965) in calves which had scoured 'for a few days.' Kenworthy (1970) attributed the changes he observed to impaired nutrition of epithelial cells, but increased migration rate may have also played a part.

In earlier work Kenworthy, Stubbs and Syme (1967) showed that the intestinal mucosa of piglets showed progressive ultrastructural changes with age, which were exacerbated at weaning. The changes were particularly severe in cases of diarrhoea. These findings are in keeping with the contention (Sprinz, 1962) that the normal gut is in a state of 'physiological inflammation', and it seems possible that if a similar situation exists in calves then exacerbation of this inflammation may be either a cause or result of diarrhoea.

Useful information on the relationship between mucosal changes and luminal bacteria can be obtained by studying changes in germ free animals after introducing known strains of micro organisms. So far this type of work has been mainly restricted to small laboratory animals and piglets since calves are difficult to produce and maintain in the germ-free state. However, work done using germ-free piglets has some relevance.

Kenworthy (1970) showed that when gnotobiotic piglets three weeks of age were contaminated with a pathogenic strain of E.coli (0141 K85 a, c(B)) there was an acute exudative inflammatory response which differed markedly from that seen with 'non-pathogenic' strains. In the latter case the mucosal changes were said to be relatively slight. With the pathogenic strain however, he was unable to show penetration of cells by micro-organisms, and the microvilli remained morphologically undisturbed by the close association with the pathogen. This contrasts with the finding of Staley, Jones & Corley (1969) who studied the effect of a different strain of E.coli (055 B5 H7) in neonatal germ-free piglets. These authors found considerable penetration of organisms into the mucous membrane with severe disruption of microvilli.

The difference between these results and those of Kenworthy (1970) is

possibly one of age, since the neonatal piglets would be in the stage when the intestine could absorb macromolecular protein, and so may also have been more susceptable to invasion by microorganisms. There may also have been greater invasiveness associated with the particular organism used.

Where there is damage to the microvilli as described by Staley et al (1969) the capacity of the intestinal mucosa to digest and absorb nutrients seems almost certain to be impaired. Where microvilli remain intact, as in the animals described by Kenworthy (1970) then the interference with cellular function is less obvious. However the other features described -exudation and desquamation- suggest that digestion and absorption may also be affected in cases such as this.

In the presence of such interference with normal epithelial digestive function, undigested food material is likely to reach the lower gut where it may be digested by microorganisms, so producing a fermentative or putrefactive diarrhoea. (Weijers and Van der Kramer, 1963).

Descriptions of the microscopic and ultramicroscopic lesions in calves are less complete than in piglets. Such descriptions stress the lack of microscopic changes associated with scour. (Ingram, 1959).

It appears that in the colostrum fed calf with diarrhoea, penetration of organisms into the mucous membrane is uncommon. In this case it seems that if E. coli plays a role in the disease, even if that role is a secondary one, there are probably one or more humoral agents produced by the organisms in the intestinal lumen which affect the intestinal mucosa in such a way as to impair its ability either to digest nutrients, or to absorb fluid, food and electrolytes. Alternatively such humoral agents may produce a secretory response in order to cause the net loss of fluid typical of the condition.

4. Bacterial products possibly associated with the diarrhoea syndrome in calves.

A. Bacterial endotoxin

A lipopolysaccharide component of the cell wall of a number of gram-negative bacteria has been shown to produce toxic effects in animals when injected intravenously (Raskova & Vanecek, 1964). Among the effects seen are pyrexia, diarrhoea, prostration and death (Wilson & Miles, 1964). However, the properties of bacterial endotoxin from strains associated with calf diarrhoea could not be differentiated from those of other non-pathogenic strains (Harvey & Carne, 1960).

It has been suggested that occasionally endotoxin may be absorbed from the gastro-intestinal tract of calves, and may be responsible for the circulatory collapse seen in 'enteric-toxaemic' forms of scour in calves (Gay 1965; Penhale 1965). The factors responsible for the sudden absorption in such cases are not clear.

The role, if any, of bacterial endotoxin in producing the intestinal manifestations of diarrhoea in the enteric form of scour is poorly defined. It seems that intraluminal administration of endotoxin in most species has no effect (Sprinz, 1969). However, Wray (1969) states that oral administration of 10-25 mg amounts of endotoxin to calves produced systemic effects (dyspnoea and abdominal discomfort,) while on post-mortem, hyperaemia of the intestine was seen. This statement is in agreement with the marked sensitivity of calves to the systemic effects of endotoxin (Penhale, 1965).

It has been suggested that some of the intestinal lesions seen in calves suffering from diarrhoea may result from hypersensitivity reactions to the endotoxin

(Wray and Thomlinson, 1969b). This suggestion was based on the similarity between the lesions of experimental hypersensitivity and those seen in clinical cases. However, Sojka (1965) commenting on somewhat similar conclusions of Thomlinson and Buxton (1962) concerning oedema disease in piglets, draws attention to the feature of biological systems that structural and functional changes of marked similarity may be governed by quite different mechanisms.

B. Bacterial products derived from food material.

In the normal animal, digestion and absorption in the upper small intestine ensures that comparatively little undigested food material reaches the distal ileum and large intestine. If for some reason, large amounts of protein or carbohydrate reach the lower gut, then the products of bacterial action may become toxic.

(a) Protein breakdown products.

Abnormally high amounts of protein reaching the lower intestine may result from a deficiency of proteases, decreased absorption of breakdown products, or an abnormally rapid propulsion of digesta allowing insufficient time for digestion (Weijers and Van der Kramer, 1963).

In relation to protease deficiency, it has been shown that piglets suffering from gastroenteritis had less protease in their intestinal content than normal pigs (Juhasz, Tamasi and Pesti, 1967). This was not simply a reflection of the greater amount of fluid entering the lumen and causing dilution of the contents, since even on a dry matter basis, there was significantly less protease in the jejunal contents of diseased animals. Blaxter & Wood (1953) showed that large losses of protein were occurring in the faeces of scouring

calves, and suggested that considerable breakdown of protein by bacteria was occurring.

The products of bacterial breakdown of proteins will include polypeptides, amino acids and ammonia. Some of the amino acids may be further converted to amines, which are particularly likely to produce pharmacodynamic effects either locally, or systemically if absorbed.

(i) Histamine. Histidine may be decarboxylated to histamine by histidine decarboxylase formed by E. coli and other intestinal organisms (Parrot & Nicot, 1966). It has been suggested (Pickrell, Rhoades, Gossling and Link, 1968) that this process may be implicated in the pathogenicity of some strains of E.coli., since they showed that while seven strains of E. coli associated with enteric disease in piglets produced histidine decarboxylase in vitro, one strain from a normal animal did not. In calves, high levels of histamine activity have been recorded in faeces from scouring animals and the amounts found were proportional to the severity of the condition (Kovacevic, 1965).

The significance of intraluminal histamine is not clear, since a part at least will be oxidised by mucosal diamine oxidase or acetylated by intestinal microorganisms (Schayer, 1966). Moreover, intestinal mucus may play a role in inactivating luminal histamine (Parrot & Nicot, 1966). If histamine reaches the systemic circulation, then an increase in coordinated waves of intestinal contraction may be expected (Bywater, 1969) although it has not been shown that increased motility is seen in diarrhoea in calves, and it has even been suggested (Smith, 1962) that motility may be decreased.

Histamine may affect the function of the intestine other than through motility, since it has been shown to affect gut enzymes by a mechanism which

may involve tissue asphyxia (Chanel and Cordier, 1958).

Endotoxin has been linked with histamine. Greisman (1960) showed that endotoxin could cause release of histamine from normal rat plasma, while Wray & Thomlinson (1969b) suggested that hypersensitivity to endotoxin was one possible mechanism of pathogenesis of scour in calves, thus implying that histamine release may be important. Furthermore Reilly and Schayer (1968) showed that endotoxin is one of the most powerful activators of histidine decarboxylase which then produces high local concentrations of 'inducible' or non-mast cell histamine. The significance of this would depend largely on the amount of luminal endotoxin entering the mucosa, and this is not yet clear.

(ii) Other Diamines. Amino-acids other than histidine may be broken down by intestinal microorganisms to produce potentially toxic amines. Thus arginine may be converted to the diamine putrescine, and lysine converted to cadaverine (Michel, 1968). This process occurs normally in the large intestine, but it has been shown that amine production in the small intestine of pigs with diarrhoea is greater than in normal animals (Porter and Kenworthy, 1969; Hill, Kenworthy & Porter, 1970). The principal amines produced were putrescine and cadaverine. These authors suggested that the amines produced in the small intestine may exert a pharmacodynamic effect in this region. It was also suggested that these amines might be responsible for the temporary malabsorption of fats and carbohydrates which had been described previously (Kenworthy & Allen, 1966).

A possible connection between diamine production and the toxicity of histamine has been suggested by Parrot & Nicot (1966). They concluded from

work with laboratory animals that under normal circumstances, only a very small proportion of histamine is absorbed in an unchanged form, the rest is either inactivated or is retained in the intestines. They found that, in the guinea-pig, mucus appeared to bind histamine and so acted as a barrier between the lumen and the intestinal wall. The diamines putrescine and cadaverine were shown to inhibit this effect, so allowing relatively low amounts of histamine in the lumen to be absorbed and produce systemic effects. This has not been demonstrated as an important factor in disease conditions, nor is there any information concerning similar effects in calves or piglets.

(b) Carbohydrate breakdown products.

Lactose is the main source of carbohydrate in the diet of the young calf, and in the healthy animal, lactose is hydrolysed by lactase in the small intestinal mucosa to glucose and galactose which are then absorbed (Huber, Jacobson, McGilliard & Allen, 1961).

Lactase deficiencies have been described in man (Davidson, 1967) and in calves with 'digestive tract necrosis', (Kwiatkowski, 1967.) Where a lactase deficiency exists, then excessive undigested carbohydrate is likely to reach the large intestine, where it will be fermented by bacteria producing large amounts of volatile fatty acids (Weijers and Van der Kramer, 1963). Increased amounts of volatile fatty acids in the faeces of scouring calves have been described by Blaxter and Wood (1953) suggesting that a fermentative process was occurring in the animals which they examined.

The significance of the bacterial products may be twofold. Firstly, the bacterial multiplication together with the production of low molecular weight

products, may, by increasing the osmolality of the gut contents, tend to draw fluid into the lumen (Christopher and Bayless, 1968). Secondly the volatile acids may produce irritation of the gut wall, and so produce diarrhoea (Weijers and Van der Kramer, 1963). This requires further proof (Fordtran, 1966), and it has been suggested that the osmotic effect is sufficient in itself to account for the diarrhoea (Christopher & Bayless, 1968).

C. Escherichia coli enterotoxin/s

De, Bhattacharya and Sarkar (1956) showed that cultures of certain strains of E.coli isolated from disease in man could cause dilatation when placed in ligated loops of rabbit intestine. This was confirmed by Taylor, Maltby and Payne (1958). Taylor et al.(1958) made an unsuccessful attempt to produce dilatation with cell-free material obtained from cultures of E.coli.

Taylor and Bettelheim (1966) found that dilatation of rabbit loops could be produced by chloroform-killed cultures of human origin which caused dilatation when living. Moreover, strains which did not cause dilatation when live did not do so after being killed by chloroform. The toxic factor which appeared responsible for the dilatation was found to be very unstable and lost potency when stored or when residual chloroform was removed by bubbling air through the mixture.

Smith and Halls (1967b) were successful in their attempts to produce dilatation of ligated loops of intestine in calves and piglets using cell free filtrates of soft agar cultures of organisms which produced dilatation in the live state. They suggested therefore that an enterotoxin was produced by these organisms. This material was found to be non-dialysable, non-antigenic, and to be stable when heated at 100°C for 30 mins. The

stability seemed to indicate that this material differed from that described by Taylor and Bettelheim (1966); moreover, it did not appear to resemble endotoxin, since the latter substance did not cause dilatation of ligated loops. Smith and Halls (1968) showed that the ability to produce enterotoxin was transmissible between strains of E.coli.

Truszczynski and Pilaszek(1969) repeated some of the observations of Smith and Halls (1967b) in piglets. They found that a larger proportion of their animals appeared insensitive than had been found by Smith and Halls, although the same method of enterotoxin production was used. Moreover, they found that heating at 100°C for 30 minutes caused inactivation of their material.

Kohler (1968) studied enterotoxic activity by administering culture filtrates intragastrically to young piglets, instead of the ligated loop technique. He observed the presence or absence of diarrhoea 3 - 10 hrs after administration and found that this method gave results which agreed with those from ligated loop experiments. Smith and Halls (1968) found no signs of diarrhoea in piglets or calves after oral administration of enterotoxic filtrates, although later Smith & Gyles (1970) reported that oral administration of filtrates from other enterotoxic strains caused diarrhoea in piglets, and concluded that there was probably a low concentration of active material in the filtrates used by Smith and Halls (1968). Diarrhoea in calves after oral enterotoxin administration has also been described (Wray and Thomlinson, 1969a).

Gyles and Barnum (1969) used ligated loops in pigs to investigate an enterotoxic material recovered from lysates of enteropathogenic strains of E.coli. This differed from the heat stable, non-antigenic, principally

extracellular material described by Smith and Halls (1967b). In contrast the enterotoxin described by Gyles and Barnum (1969) was found to be heat labile, antigenic and principally intracellular. A particularly interesting observation was that the heat labile enterotoxin appeared to be antigenically indistinguishable from the enterotoxin of Vibrio cholerae.

Both heat labile and heat stable enterotoxins are governed by intracellular plasmids, and so are transmissable (Smith and Gyles, 1970). In a series of transmission experiments, Smith and Gyles were unable to transmit the ability to produce one toxin without transmitting the other. They suggested therefore that, despite the apparent differences in their characteristics, the two toxins were probably two forms of the same enterotoxin, and suggested that the heat stable fraction may be combined with a protein molecule to form the heat labile toxin.

D. Other bacterial products.

a) Haemolysins.

Haemolysin production is a common feature of strains of E.coli associated with diarrhoea in pigs (Gay, 1965). In calves, however, there appears to be no correlation between haemolysin production and enteropathogenicity.

b) Mucinase.

Ross (1959) found a higher mucinase production by strains of E.coli associated with enterotoxicity than by strains from normal infants. However, Formal, Lowenthal and Galindo (1958) found considerable mucinase production in a strain thought to be non-pathogenic for man, while other strains considered pathogenic were found to produce little. Moreover, Freter (1955) was unable to link mucinase production by strains of Vibrio cholerae with virulence, although he

found that the mucinase titre could vary within strains, and was also dependant on the medium used. Hoskins and Zamcheck (1968) showed that mucinase production occurred *in vivo* by comparing faeces of germ-free and conventional rats. They concluded that the micro-organisms in normal animals caused breakdown of intestinal mucins.

The precise role of mucus is uncertain (Schrager, 1970) but it is generally considered to have a protective function (Hendrix and Bayless, 1970). If this is the case, then it is possible that mucinase production may enhance enteropathogenicity of *E.coli*.

Conclusions.

It appears likely that bacterial products are of importance in the pathgenesis of diarrhoea in calves. Of the products mentioned, bacterial endotoxin seems unlikely to be a major primary factor, since it is present in the gut of healthy animals, and material from pathogenic strains of *E.coli* seems indistinguishable from that derived from non-pathogenic strains.

However, enterotoxin production seems likely to be important in at least some cases of diarrhoea in calves, and to be worthy of further investigation.

Malabsorption of protein and carbohydrate food material, with subsequent fermentation by microorganisms, seems a possible factor in calf diarrhoea which in the past has received little attention.

Other bacterial products such as mucinase and haemolysin may have a secondary role, but seem likely to be of only minor importance.

Purpose of the present experiments.

1. To examine the pharmacodynamic activity of enterotoxic material on isolated preparations.
2. To attempt to develop a more convenient and sensitive test preparation than the ligated loop for demonstrating enterotoxin activity on calf intestine.
3. To study some of the effects of enterotoxin on intestinal absorptive activity.
4. To look for changes in lactase activity in intestinal mucous-membrane of calves with diarrhoea, and for any effect of enterotoxin on the activity of the enzyme.

SECTION I

EXPERIMENTS TO CONFIRM THE ACTIVITY OF ENTEROTOXIN IN LIGATED LOOPS OF CALF INTESTINE AND INVESTIGATE ITS ACTIVITY IN OTHER EXPERIMENTAL SYSTEMS.

INTRODUCTION.

Enterotoxicity of strains of E.coli. for calves has been shown by the capacity of cultures or culture filtrates of these strains to produce dilatation of ligated loops in calf intestine (Smith and Halls, 1967 a & b). Some enteropathogenic strains of E.coli. were kindly supplied by Dr.Williams Smith, and it was first necessary to confirm that culture filtrates from these strains produced dilatation of ligated loops of calf intestine.

The mode of action of the gut dilating factor is unknown. However, if activity could be demonstrated in other experimental systems, it was felt that this might provide useful information on the mode of action in the intestine and may also provide an assay method more convenient than ligated loops.

One possible effect of enterotoxic material may be to increase the permeability of mucosal blood vessels. This may be demonstrated by intradermal injection, either alone or after intravenous injection of a protein-bound dye. Cholera enterotoxin has been examined in this way, and has been shown to contain a factor which increases blood-vessel permeability in the skin of guinea-pigs (Craig, 1965) and which appears to be closely related to the choleragenic factor

(Finkelstein and Lospalluto, 1969). A skin-permeability effect has been described for E.coli culture filtrates on intradermal injection in calves (Wray and Thomlinson, 1969a). It seemed of interest to seek to confirm this report.

The short-circuited frog skin preparation has been used as an indication of active sodium transport (Ussing and Zerahn, 1951). It has been shown that filtrates of cultures of V. cholerae cause a sharp reduction of the short-circuit current when placed in contact with the skin (Fuhrman & Fuhrman 1960). This led to the suggestion that inhibition of active sodium transport might play an important role in the fluid production associated with cholera (Phillips 1964).

However, it was later shown that the sodium pump inhibitor could be separated from the factor responsible for diarrhoea (Burrows, Musteikis, Oza and Dutta, 1965). This has led to the unpopularity of the sodium-pump inhibition theory, and it has been suggested that the presence of ammonia in the preparative procedure may have caused an artifact (Burrows, 1968).

Despite this position with regard to cholera toxin, it seemed worthwhile examining the effect of coli enterotoxin on short-circuit current in the frog-skin preparations to seek possible effects on active transport mechanisms.

The Adenosine triphosphatase which is stimulated by sodium and potassium ions /($Na^+ + K^+$) ATPase/ and which is inhibited by ouabain is widely assumed to be involved in active transport mechanisms (Skou,1965). Certain purgative drugs such as phenolphthalein have been shown to inhibit ($Na^+ + K^+$) ATPase from intestinal mucosa of the rat (Chignell,1968) and rabbit (Richardson,1968). The latter author found that culture filtrates of Vibrio cholerae also caused

inhibition of the enzyme, and it was suggested that inhibition of ($Na^+ + K^+$) ATPase might be a factor in the diarrhoea associated with purgation and with cholera.

It therefore seemed of interest to know whether bovine intestine possessed ($Na^+ + K^+$) ATPase, and whether the enzyme is affected by enterotoxic culture filtrates.

Mucinase production has been suggested as a possible factor affecting virulence of strains of E.coli associated with infantile gastro-enteritis (Ross, 1959). The enterotoxic culture filtrates were therefore examined to determine whether they showed significant mucinase activity.

Certain strains of E.coli produce histamine by decarboxylation of histidine (Parrot and Nicot, 1966; Pickrell, Rhoades, Gossling and Link, 1968). It is therefore possible that histamine may be present in culture filtrates of enterotoxic strains of E.coli and may be demonstrated by its effect on isolated guinea-pig ileum. Alternatively, other substances involved in enterotoxicity might be detectable by their effect on isolated smooth muscle preparations.

EXPERIMENTAL METHODS AND RESULTS.

1. Confirmation of Activity in Ligated Loops.

Methods.

a) Culture filtrate preparation.

The organisms tested were those designated B41 (O101:K ?); B42 (O9:K9); B44 (untypable); and S13 (O8:K ?). These had all been found to produce dilatation of ligated loops of calf intestine (Smith and Halls, 1967a). A commensal strain was also tested. The organisms were taken from Dorset egg slopes and were plated out on blood agar. After overnight incubation, single colonies were subcultured in glucose broth and incubated for six hours. After this time, 1 ml amounts of the glucose-broth culture were added to Roux flasks containing 200 ml of the semi-solid agar medium described by Smith and Halls (1967b). The Roux flasks were placed on their sides and incubated for 18 hours at 37°C.

At the end of this time, the flasks were cooled to 4°C, and the medium was filtered through two layers of muslin. The resulting filtrate was centrifuged at 12,000 r.p.m. to separate the microorganisms, and the clear supernatant was heated at 65°C for 10 minutes to kill any remaining organisms.

Uninoculated culture medium was treated in an identical manner as a control.

b) Preparation of acetone extracts of culture filtrates.

Acetone extracts were prepared by adding 8 volumes of acetone to the culture filtrates prepared as described above. After standing overnight at -30°C, the clear supernatant was decanted, and the precipitate dried by blowing air over it.

c) Preparation of ligated loops.

The technique for the preparation of ligated loops was similar to that described by Smith and Halls (1967a) with two exceptions:-

1) The calves (designated 1 and 2) were anaesthetised throughout the period of the experiment.

2) The time allowed for dilatation to occur was 10 hours instead of 24 hours.

Anaesthesia was induced by i/v administration of pentobarbitone sodium (15 mg/kg) and maintained by further dosage as necessary. The abdomen was opened, and the duodenum located. Starting approximately 2.5 m from the pylorus, loops of intestine about 10 cm in length were tied off using silk ligatures. Loops were alternately injected with 15 ml of culture fluid to which neomycin (100 µg) had been added, or were left uninoculated. 37 loops were produced in all in calf 1 leaving the last 3 m uninjected. Culture fluid from each organism was injected at least three times, and the sites of inoculation of each were spaced along the intestine to allow for variation in susceptability of the different regions.

At the end of the experiment, the animal was killed by i/v injection of pentobarbitone and the abdomen opened. Each loop was drained and the volume of contents measured. The loop was then opened along its length, blotted dry and weighed. The volume of exudate was then expressed as the volume per 10gm (wet weight) of intestine.

In the second animal (calf 2) 15 ml amounts of culture filtrate from strain B44 alone were tested, and compared with uninoculated culture medium. Acetone extracts derived from 15 ml of culture filtrate were redissolved in 15 ml of

saline, and compared with extracts of uninoculated culture medium.

d) Lactase and Alkaline Phosphatase activity in mucosa of ligated loops.

Samples of mucosa from loops of calf 2 were preserved for mucosal enzyme determination. Lactase was estimated as described later (section 4). Alkaline phosphatase was estimated by the method of King and Wootton (1964).

Results.

Table (1) shows the results of ligated loop experiments in calf 1, in which the strains B41, B42, B44, and S13 were tested and compared with the effect of uninoculated culture medium and with material from cultures of a commensal strain of E.coli. Strains B41, B42 and B44 produced a greater exudate than either strain S13, the commensal strain or the control. Strain B44 appeared the most active.

Organism	No. of loops	Mean vol of exudate per 10 gm of intestine
B41	3	40.6
B42	3	39.3
B44	3	45.0
S13	3	25.0
Commensal	2	33.0
Uninoculated culture medium	6	31.0
Control loops (uninoculated)	18	18.2

Table(1.)Volume of exudate in ligated loops (calf 1).

The effect in the second calf of B44 culture filtrates and acetone extracts derived from them is shown in Table (2). The activity of culture fluid was significantly greater than that of uninoculated culture medium ($p<0.01$, Mann-Whitney U-test, (Siegel, 1956)). The acetone extract derived from B44 culture filtrates retained much of the enterotoxic activity.

Material	No. of loops	Mean vol. of exudate per 10 gm of intestine	
B44 culture filtrate	5	49.8)	$p< 0.01$*
Uninoculated culture medium	4	29.0)	
B44 acetone extract	2	38.5	
Uninoculated culture medium acetone extract	2	17.0	
Uninoculated loops	14	17.3	
		* Mann-Whitney U-test	

Table (2) Volume of exudate in ligated loops. (calf 2)

The lactase and alkaline phosphatase activity in mucosal samples from ligated loops in Calf 2 are shown in Table 3. In neither case was there any significant difference between the groups.

	Lactase	Alkaline Phosphatase
Loops containing B44 culture filtrate (5)	11.7 ± 0.7	2.0 ± 0.55
Loops containing Uninoculated culture medium filtrate (4)	9.4 ± 2.4	2.16 ± 0.75
Uninjected loops (5)	8.8 ± 3.3	1.98 ± 0.95

Table(3.) Lactase (μM Lactose hydrolysed/10mg mucosa/hr) and alkaline phosphatase (King Armstrong units/g of mucosa) in mucosa from ligated loops of calf 2.

2. The effect of intradermal injection of culture fluids in guinea-pigs and calves.

Methods.

a) Experiments using guinea-pigs.

Albino guinea-pigs were prepared by clipping and depilating both flanks and abdomen. Pontamine sky blue (7.5 mg per kg) was injected intravenously, and this was followed by intradermal injection of 0.1 ml amounts of culture filtrate. The filtrate produced from the strains B41, B42, B44, uninoculated culture medium and saline were injected using a Latin square arrangement.

b) Experiments using calves.

Calves were prepared by clipping and depilating areas on the flank, and by observing changes after intradermal injection of 0.1 ml amounts of material. As well as culture filtrate, acetone extracts were used after being redissolved in saline.

Results.

a) Guinea-pig experiments.

With the exception of the site of the saline control, bluing appeared around each site of injection within 30 minutes. It was not found possible in any of four animals to distinguish the reaction resulting from the injection of uninoculated culture medium filtrate from that due to enterotoxic filtrate.

b) Calf experiments.

The area around the injection sites showed a progressive oedematous reaction in response to enterotoxic culture filtrate, and to acetone extracts thereof. However, uninoculated culture medium was again found to cause a reaction in comparison with the saline control. Table (4) shows the results obtained in a typical experiment. A preparation of endotoxin (E.coli lipopolysaccharide) was also injected for comparison.

Site	1	2	3
B44 culture filtrate	++	+	+
uninoculated medium filtrate	++	+	+
B44 acetone extract	+	++	++
uninoculated medium acetone extract	+	+	+
Saline	-	-	-
E,coli lipopoly-saccharide 100µg	++	N.T.	N.T.

N.T. not tested
- no reaction
+ mild "
++ moderate "

Table(4.) Skin reactions 1 hr after intradermal injection in a calf.

3. Effect of enterotoxin containing material on short-circuit current in frog skin.

Methods.

The mode of preparation of the frog skin and the apparatus used was largely as described by Ussing and Zerahn (1951). After allowing the preparation to stabilise, the short-circuit current was measured at 5-minute intervals. Culture filtrate was added to either the inside or the outside of the skin, and the effect on short-circuit current was observed. Acetone extract redissolved in Ringer solution was added to the preparation in a similar manner. In each case the effect seen was compared with that produced by equivalent amounts of material from uninoculated culture medium.

Results.

The effect of 12.5 ml and 25 ml of culture filtrate was tested on both sides of frog skin preparation and was found to have no effect on the short-circuit current that could be distinguished from the control material. Similarly, weighed amounts of acetone extract were found to produce only minor effects which were again indistinguishable from effects produced by control material. Five preparations were used in these experiments, and all gave results similar to those shown in Fig. (1).

4. The effect of E.coli enterotoxin on ouabain-sensitive Adenosine Triphosphatase.

Methods.

Bovine intestine was taken from 4-7 day old dairy bull calves or from adults within five minutes of slaughter at the abattoir. Samples were taken from the jejunum about 3m from the duodenum, and were washed and carried to the laboratory in iced saline. Rat jejunum was removed from freshly killed animals.

Mucosa was removed by gently scraping with the edge of a microscope slide. The homogenisation medium contained 12mM Tris-2mM EDTA (pH 7.6). A wet weight of approximately 60-100mg was homogenised in 5 ml of the above medium in an all glass homogeniser by 25 strokes of the plunger.

ATP-ase activity was assayed in most experiments using the method described by Hirschhorn and Rosenberg (1968). The homogenate of mucosa was spun at 850g for 20 minutes at 5°C, the supernatant was discarded and the sediment

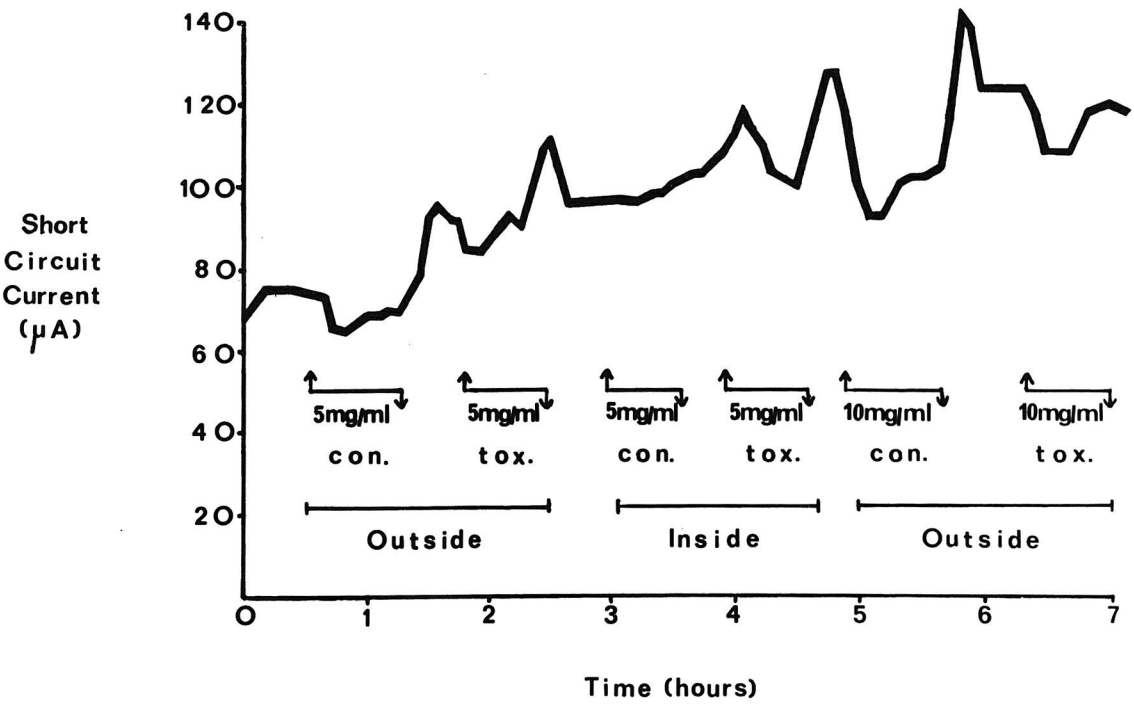


Fig 1. Short-circuit current across frog skin. Enterotoxic (tox.) and control (con.) extract was added at the points shown to fluid bathing either inside or outside of the skin.

resuspended in 9 volumes of 12mM Tris-2mM EDTA at a pH of 7.6. 0.1 ml amounts of the homogenate were then incubated with 2.4 ml amounts of a medium containing 4mM magnesium chloride; 140mM sodium chloride; 16mM potassium chloride; 30mM Tris; 2.5mM ATP, (Tris or di-sodium salt). The pH of the incubation mixture was 7.4. The reaction was stopped by the addition of 0.5ml of 50% Trichloracetic acid (Brown, Smith and Witty, 1968) since it was found that addition of perchloric acid as used by Hirschhorn and Rosenberg (1968) could release phosphate from ATP.

In some experiments, the assay was carried out as described by Brown, Smith and Witty (1968). In this case, the homogenising medium consisted of 0.25M sucrose containing 5mM EDTA, sodium deoxycholate 0.1% and 60mM histidine at pH 7.1. The incubation medium in this case contained 6mM magnesium chloride; 100mM sodium chloride; 20mM potassium chloride; 10mM ATP and 10mM histidine.

In either method the inorganic phosphate released was estimated using the method of Fiske and Subbarow (1925). Protein was estimated by the method of Lowry, Rosenbrough, Farr and Randall, (1951) using crystallised bovine albumin as standard.

ATP-ase activity was expressed as μM of inorganic phosphate liberated per mg. protein per hour (μMPi/mg prot/hr).

Tris-ATP, disodium ATP, ouabain and albumin were obtained from Sigma Chemical Co; St. Louis U.S.A. Other reagents were A.R. grade and obtained from British Drug Houses Ltd.

The effect of enterotoxic culture filtrates from strain B44 were tested on preparations of rat intestinal mucosa which contained substantial amounts of $(Na^+ + K^+)$ ATP-ase. In these experiments 0.1 ml amounts of culture

filtrate containing enterotoxin activity were added to the incubation mixture and pre-incubated for five minutes before adding ATP.

Results.

a) Ouabain-sensitive ATP-ase in bovine intestinal mucosa.

Only a small percentage of the total ATP-ase in bovine mucosa was found to be inhibited by 10^{-3}M ouabain. When the same technique was used for rat mucosa, the proportion of ouabain-sensitive enzyme was greater, but the total amount of enzyme present was less (Table 5). Adult intestinal mucosa also showed very small amounts of ouabain-sensitive enzyme.

When the method of Brown, Smith and Witty (1968) was used on calf mucosa the results were similar to those above.

b) The effect of enterotoxic culture filtrate on rat mucosa.

The presence of enterotoxic culture filtrate in the amounts used did not significantly affect the activity of ouabain-sensitive rat ATP-ase (Table 6).

	Total ATP-ase (μMPi/mg prot /hr)	Ouabain-sensitive ATP-ase (10^{-3}M)	Percentage inhibition	No of observations	Method
CALF	27.2±4.6	1.9±0.5	7.0	13	Hirschhorn & Rosenberg 1968
ADULT BOVINE	15.7	0.4	2.5	3	"
RAT	6.9±1.0	3.3±0.9	48	10	"
MAN *	9.7	4.1	42	3	"
CALF	30.7±5.2	1.9±0.9	6.2	4	Brown et al 1968
PIGLET XX (1-7d.o.)	32	(10^{-4}M ouabain)	25	1-7	"

* Results of Hirschhorn & Rosenberg 1968.

XX Results of Brown et al.1968.

Table (5) Total and ouabain-sensitive ATP-ase activity in bovine and rat intestine (Means ± S.E.) Figures for man and pig are included for comparison.

	Total ATP-ase	ATP-ase + enterotoxin	ATP-ase + ouabain (10^{-3}M)	No. of observations.
ATP-ase (μMPi/mg prot/hr.)	5.3 ± 0.8	5.1 ± 0.8	3.1 ± 0.7	7

Table (6). The effect of enterotoxin and ouabain on ATP-ase from rat intestine.

5. Mucinase activity of enterotoxic culture filtrates.

Methods.

In vitro mucinase activity was measured by the ability of mucinase to depolymerise ovomucin so that the latter was no longer precipitated by cetyl trimethyl ammonium bromide (CTAB) (Formal, Lowenthal and Galindo, 1958). Ovomucin was prepared as described by Freter (1955) and the concentration was such that dilution 1:2 with normal saline did not interfere with the formation of a precipitate with CTAB, while a dilution 1:4 prevented precipitation.

Culture filtrates and control material were diluted serially with borate-buffered saline (pH 7.0) so that the final volume was 0.5 ml in each case. To each tube, 1.0 ml of the ovomucin preparation was added, and the tubes were incubated at 37°C for 30 minutes.

After incubation, three drops of CTAB were added to each tube, and the presence or absence of precipitate was noted. The mucinase titre was taken as being the highest dilution at which addition of CTAB failed to produce precipitation of ovomucin.

Results.

Results are shown in Table (7). The titres of the enterotoxic filtrates were similar to the uninoculated control medium and to non-pathogenic strains and were all very much less than those found by Ross (1959) in strains of E.coli associated with diarrhoea in infants.

Organism	Titre.	No. of expts. in which result was obtained.
Uninoculated culture medium(control)	1:2	3
	1:4	1
B44 (filtrate unheated)	1:2	2
	1:4	1
	1:8	1
B44 (filtrate heated to 100°C for 15 min.)	1:4	1
Commensal strain E.coli	1:2	1
	1:4	1
	1:8	1
E.coli 078 K80	1:2	1
E.coli strain. P307 Ent. -ve	1:4	1
E.coli strain. P307 Ent. +ve	1:2	1

Table (7). Mucinase titres of culture filtrates from different strains of E.coli.

6. The effect of enterotoxic culture filtrates on isolated guinea-pig ileum.

Methods.

Segments of distal ileum from freshly killed guinea-pigs were mounted in a 10 ml organ bath containing oxygenated Tyrode solution at 37°C. Contractions were recorded using an isotonic lever and kymograph. Drugs were added at regular intervals, in volumes of less than 0.2 ml, and the preparation was washed between responses.

After allowing time for the preparation to settle, a dose-response curve to histamine was established, and afterwards the experiment was limited to the section of the dose-response curve which lay between 30 and 70 per cent of the maximal response.

Histamine assay was carried out using bracket (3-point) assay to compare unknown solutions with standard doses of histamine. The histamine content of enterotoxic and control solutions was also determined after removal from Thiry-Vella loops.

Results.

It was found that both the enterotoxic culture filtrates and the control filtrates caused contractions of the guinea-pig ileum which were completely inhibited by 1μg/ml mepyramine (Fig 2). It therefore appeared that the contractions were due to histamine.

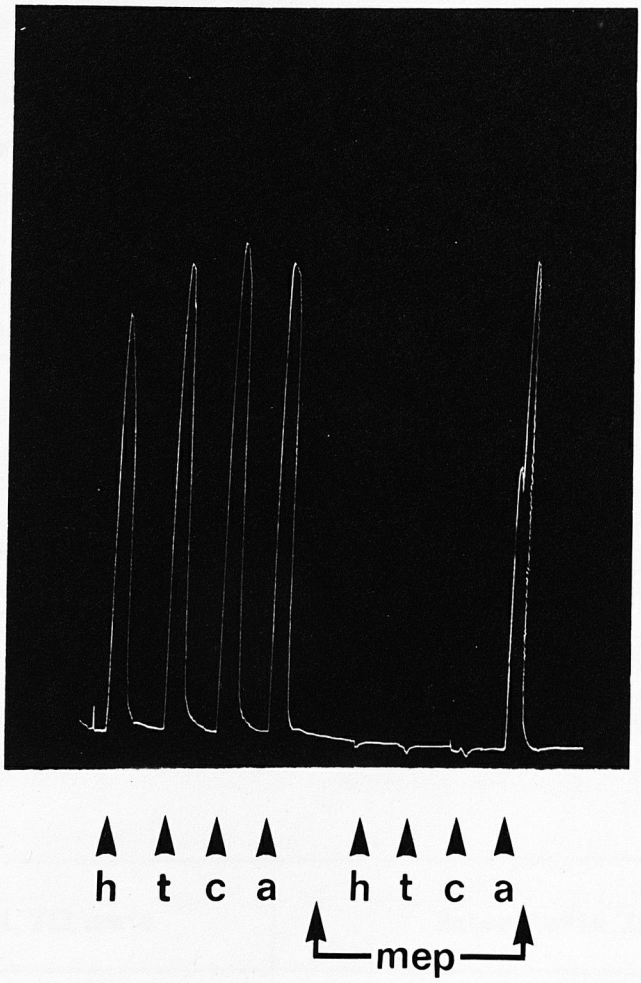


Fig.2 Responses of isolated guinea pig ileum to histamine (h,1.0μg); enterotoxic culture filtrate (t, 0.25ml); control filtrate (c,0.25ml); and acetyl choline (a, 0.5μg). Mepyramine (mep,5μg) was added as shown. Amounts refer to the dose added to a 10 ml organ bath.

Assays of histamine content of six different batches of enterotoxic and control filtrates showed that they contained similar amounts of histamine (Table 8). There was no significant difference between the two groups.

	Control filtrate	Enterotoxic filtrate
Histamine content (μg/ml)	2.5 ± 1.1	2.1 ± 0.5

Table (8). Histamine content of control and enterotoxic filtrates.

Figures represent means of six determinations ± S.E. of the mean. The difference between the groups is not significant ($p > 0.05$).

The histamine content of fluid recovered from Thiry-Vella loops after 30 mins. exposure to enterotoxin was no higher then at the start of the period. (Table 9).

	Control filtrate	Enterotoxic filtrate
Histamine content before 30 min. test period (μg/ml)	2.2	2.3
Histamine content after 30 min. test period (μg/ml)	1.2	1.1

Table (9). Histamine content of control and enterotoxic solution before and after 30 min. test periods in Thiry-Vella loops. Each figure represents the mean of two experiments.

DISCUSSION

The results of the ligated loop experiments in the first calf (Table 1) confirmed that culture filtrate from strains B41, B42 and B44 all appeared to cause accumulation of fluid in the loops when compared with the effect of uninoculated control medium or filtrates from the commensal strain. However both these latter contained greater volumes of fluid than did the uninoculated control loops. The strain S13 appeared devoid of dilating ability.

Since the strain B44 appeared particularly active, it was retested in a second calf (Table 2) and acetone extract was also tested in this animal. The activity of culture filtrate in comparison with uninoculated control medium was confirmed and shown to be significant ($p < 0.01$ Mann-Whitney U-test, Siegel 1956). The acetone extract also appeared to retain a considerable proportion of the activity, as suggested by Smith and Halls (1967b).

The strain B44 was therefore chosen as being a strong producer of enterotoxin, and was the organism used in most of the experiments to be described.

Neither lactase nor alkaline phosphatase activities were lower in the toxin-containing loops than in the controls. This suggests that major mucosal damage with associated malabsorption was not occurring.

The intradermal injection experiments showed that while culture filtrate produced a permeability response in both guinea-pigs and calves, this could not be clearly distinguished from the effect produced by uninoculated culture medium. This is contrary to the findings described by Wray and Thomlinson (1969), who found that uninoculated soft agar culture medium gave a

negative response on intradermal injection in calves, and suggested that the dermal reaction which they obtained using soft agar culture filtrates was due to the lipopolysaccharide content. It is possible that the response to intradermal injection of uninoculated culture medium obtained in the present experiments may be due to substances with a large molecular weight in the medium which caused a non-specific reaction.

The accumulation of fluid in ligated loops of intestine might have resulted from inhibition of active transport mechanisms in the intestine. Two preparations were used in an attempt to obtain evidence of an effect on active transport. In the first of these, it was found that no effect of enterotoxic material could be demonstrated on the short-circuit current across frog skin, indicating that active transport of sodium in this preparation was unaffected. This did not exclude an effect on bovine intestine, but made it less likely. This was supported by the absence of an inhibitory effect of enterotoxic culture filtrate on ouabain-sensitive ATP-ase from rat intestine. Enterotoxin was not tested on bovine ATP-ase, since the percentage of ouabain-sensitive enzyme in the material was much smaller than that found in rat mucosa, making it difficult to demonstrate any inhibition.

Comparison of the results for ATP-ase activity in the present experiments with those of other authors using similar techniques (Table 5) shows that the rat mucosa contained both total and ouabain-sensitive ATP-ase in amounts similar to those described in man (Hirschhorn and Rosenberg 1968). The total ATP-ase in calf intestine was similar to that found in the piglet intestine by Brown, Smith and Witty (1968), but these authors found a 25% inhibition of the enzyme by 10^{-4}M ouabain.

It therefore appeared that in comparison with either rat, piglet or man, only a small proportion of calf mucosal ATP-ase was ouabain-sensitive. The significance of this finding depends on the importance of the ouabain-sensitive ATP-ase in the active transport process in the bovine. This has not been established, although it has been suggested (Robinson, 1970) that ($Na^+ + K^+$) ATP-ase may not represent the only enzyme of importance in the sodium pump mechanism even in species such as the rat where copious amounts of the enzyme are available.

The relatively high amounts of total ATP-ase found in calf material in the present experiments, and in the piglet by Brown et al. (1968) may reflect age differences, since it has been shown (Bywater, 1968) that non-specific mucosal alkaline phosphatase is higher in calves than in adult cattle. The few observations made on adult material supported this suggestion, but in these cases, the ouabain-sensitive portion of the enzyme was proportionally even smaller than in calf material.

Mucinase activity was low in the samples of culture filtrate tested, all of which showed similar activity to that in control material. This suggested that mucinase activity was unlikely to be an important factor in the entero-toxic activity of culture filtrates. This does not rule out a role for the enzyme *in vivo*, since it is possible that mucinase production by the organisms tested may occur under suitable conditions either *in vitro* or *in vivo*.

The effect of enterotoxic culture filtrates on guinea-pig smooth muscle indicated that histamine content could not account for the enterotoxic effect of culture filtrates, since there was no difference between the histamine content of enterotoxic and control solutions. The content of other pharmacol-ogically active substances did not appear sufficient to affect the isolated

guinea-pig ileum in the amounts tested.

There also appeared to be no increase in histamine content of loops during exposure to toxin, so that the suggestion that hypersensitivity reactions may account for enterotoxicity (Thomlinson,1969) are not supported by this observation. However, it has been found (Aitken and Sandford,1969) that histamine is a relatively unimportant mediator of hypersensitivity responses in the bovine, and so the absence of a rise in histamine level may not be significant. Nevertheless, any dramatic rise in other mediators (kinins etc) might be expected to produce contraction of the guinea-pig ileum, and this was not seen.

The conclusion from these experiments was that further information on the nature and mode of action of enterotoxin was most likely to be obtained by examining its effects on calf intestine *in vivo*.

SECTION II

THE USE OF THIRY-VELLA LOOPS TO STUDY THE EFFECT OF E.COLI ENTEROTOXIN ON FLUID AND ELECTROLYTE MOVEMENT IN CALF SMALL INTESTINE.

INTRODUCTION.

In the studies on E.coli enterotoxins carried out by other workers, two separate methods have been used to demonstrate enterotoxicity. The first of these is the ligated loop method (Smith and Halls, 1967b; Truszczynski and Pilaszek, 1969; Gyles and Barnum, 1969). This technique appears to be more satisfactory for work in pigs than in calves (Smith and Halls, 1967b) and is expensive in experimental animal requirements. It does not allow replication of experiments using the same length of gut and is not well suited to the study of fluid and electrolyte changes in the luminal fluid.

The second method which has been used involves the intragastric administration of enterotoxic culture filtrates to young piglets, and observing the production of diarrhoea at $1\frac{1}{2}$ to 3 hours after administration (Kohler, 1968). This method appears to present considerable problems in providing adequate controls, since there must always be a risk of diarrhoea occurring for reasons unrelated to the toxin administration. Moreover, diarrhoea is likely to be the result of a complex series of events and so is probably an insensitive indicator of enterotoxin activity, particularly for use in calves, although oral administration of enterotoxic culture filtrates appeared to produce diarrhoea in this species (Wray and Thomlinson, 1969a).

In the present experiments, a preparation was required which would allow

the study of the effect of enterotoxin on absorption of fluid and solutes from the calf intestine. For the reasons given above, both methods used by other authors for the study of enterotoxin appeared unsuitable.

An alternative approach appeared to lie in the use of Thiry-Vella loops, since this preparation would allow repeated access to the lumen of the intestine, and would be more economical in experimental animals than either of the methods described above.

Since the early description of the preparation of Thiry-Vella loops in dogs (Vella, 1882) the method has been used by a number of authors to study absorption and secretion in the intestine (e.g. Johnston, 1932; Berger, Kanzaki, Homer and Steele, 1959; Annegers and Wakefield, 1962). Thiry-Vella loops have also been used to examine the effect of cholera enterotoxin on canine intestine (Swallow, Code and Freter, 1968; Carpenter, Sack, Feeley and Steenberg, 1968; Carpenter and Greenough, 1968.)

Thiry-Vella loops, while retaining intact mesenteric blood and nerve supplies, have been criticised for their lack of luminal nutrition for the mucosal cells (Levin, 1967.) This, it was claimed, might render the preparation abnormal. On the other hand, it has been stated that loops prepared in dogs show no evidence of functional or histological change over periods as long as 7 years (Berger et al, 1959.) Studies using rats have shown that the appearance of an isolated loop examined under the dissection microscope changed less with age than did neighbouring intestine (Chacko, Mathan and Baker, 1968), or even that a partial return to the neonatal pattern of villi could occur (Gleeson, Cullen, Collins and Dowling, 1970.) In a study of functional changes in rat intestine after exclusion from continuity with the rest of the bowel, it

appeared that basic ileal glucose absorptive capacity was unaffected by intraluminal nutrition, but that in the proximal small intestine there was stimulation by contact with luminal contents, (Gleeson, Cullen, Collins and Dowling, 1969).

The problem of luminal nutrition of mucosal cells could have been overcome by the use of re-entrant loops (Levin, 1967) but initial attempts to produce these gave poor results, possibly because of the design of cannula used. The main problem was leakage around the cannulae.

It seemed possible however, that any shortcomings of Thiry-Vella loops were relatively unimportant when they were used to study changes in absorptive behaviour resulting from enterotoxin activity, since control observations were made in each case.

The use of Thiry-Vella loops for absorption studies presents certain technical problems (Shields, 1964). The first problem is the difficulty of fully draining the loops at the end of an experiment, since fluid is retained in folds of mucosa. This can be overcome by the use of a non-absorbable reference marker. A further difficulty arises in sealing the ends of Thiry-Vella loops to prevent leakage. This may be overcome by the use of suitable catheters (Code, Bass, McClary, Newmum and Orvis, 1960).

1. THE EFFECT OF ENTEROTOXIN ON NET FLUID, GLUCOSE AND ELECTROLYTE MOVEMENT FROM THIRY-VELLA LOOPS.

MATERIALS AND METHODS.

The absorption of fluid, glucose and electrolytes from a Thiry-Vella loop was observed during two consecutive 30 minute periods. During the first 30 minutes a control solution was used, while during the second 30 minute period, a solution was used which contained enterotoxic material. In this way each loop was used as its own control.

Surgical Preparation of loops.

Dairy bull calves (Fresian or Ayrshire) were usually bought in the market at about 1 - 2 weeks of age. They were fed glucose and water for 24 hrs after arrival at the animal house, after which they were bucket fed on whole milk and milk substitute, (BOCM.'Gold Top') with access to hay, nuts (BOCM 'Calf-wena pencils') and water. Calves were usually operated on at about two weeks after arrival.

Calves were starved for 18 hours prior to the operation, and were pre-medicated with atropine (0.02 mg/kg). Anaesthesia was induced by halothane given by open mask, after which the trachea was intubated and anaesthesia maintained by a mixture of cyclopropane and oxygen.

The abdomen and right flank were clipped and washed with disinfectant, and a para-median incision made under aseptic conditions. The appropriate section of the small intestine (either upper jejunum or lower ileum) was then located, and six numbered bowel clamps were used to close off two 30 cm lengths of intestine. Care was taken to ensure that both loops possessed an adequate

blood supply. After tying off blood-vessels as necessary, the two loops were separated from each other and from neighbouring intestine, and the mesentery was cut towards its base as far as possible. When the lumen was opened, care was taken to avoid soiling the abdomen with intestinal contents.

The proximal and distal cut-ends of the intestine were then joined by end-to-end anastamoses. In some cases, this was done using a polyethylene former which was eventually passed in the faeces. The mesentery was closed below the anastamosis.

The two isolated loops each retained a mesenteric pedicle carrying blood and nerve supply. The ends were then delivered through four stab incisions in the flank, and were sutured in position. The abdomen was closed and sutured using catgut for muscle and fascia layers and monofilament nylon for the skin. Fig.(3) shows the diagrammatic arrangement of the loops.

Recovery during the immediately post-operative period was usually uneventful, and most calves took glucose and water by mouth within six hours. Penicillin and streptomycin (Strypen, May and Baker Ltd.) were given for three days post-operatively.

The main problem encountered in this operation was luminal obstruction resulting from adhesions which caused kinking of the intestine. Where this occurred, symptoms of obstruction were seen at about 3-4 days after the operation, and the condition was usually fatal.

Calves which survived for 7-10 days usually remained healthy until disposed of some months later. Fig.(4) shows the appearance of a calf 5 months after installation of two loops in the distal ileum (loops O and P), together with the post-mortem appearance of the loops and neighbouring intestine 7 months later.

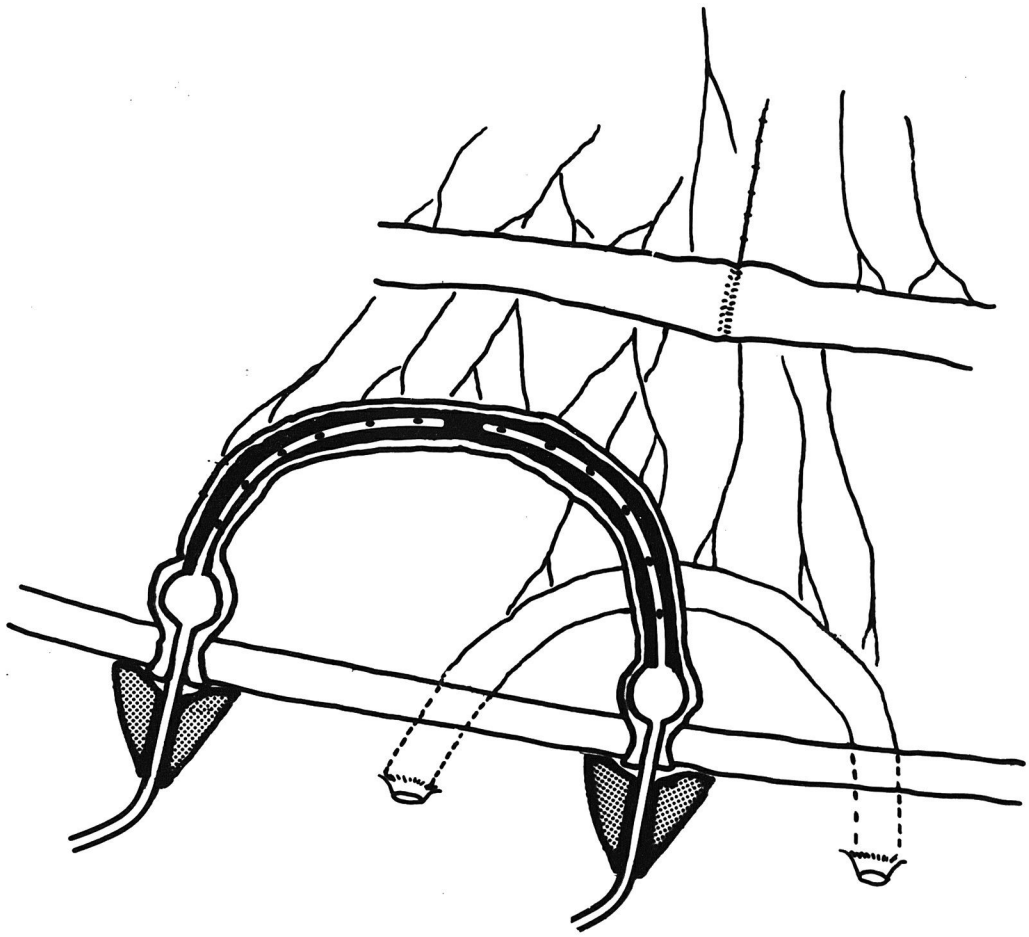


Fig.3 Diagrammatic representation of two Thiry-Vella loops, one of which shows the catheters *in situ*.

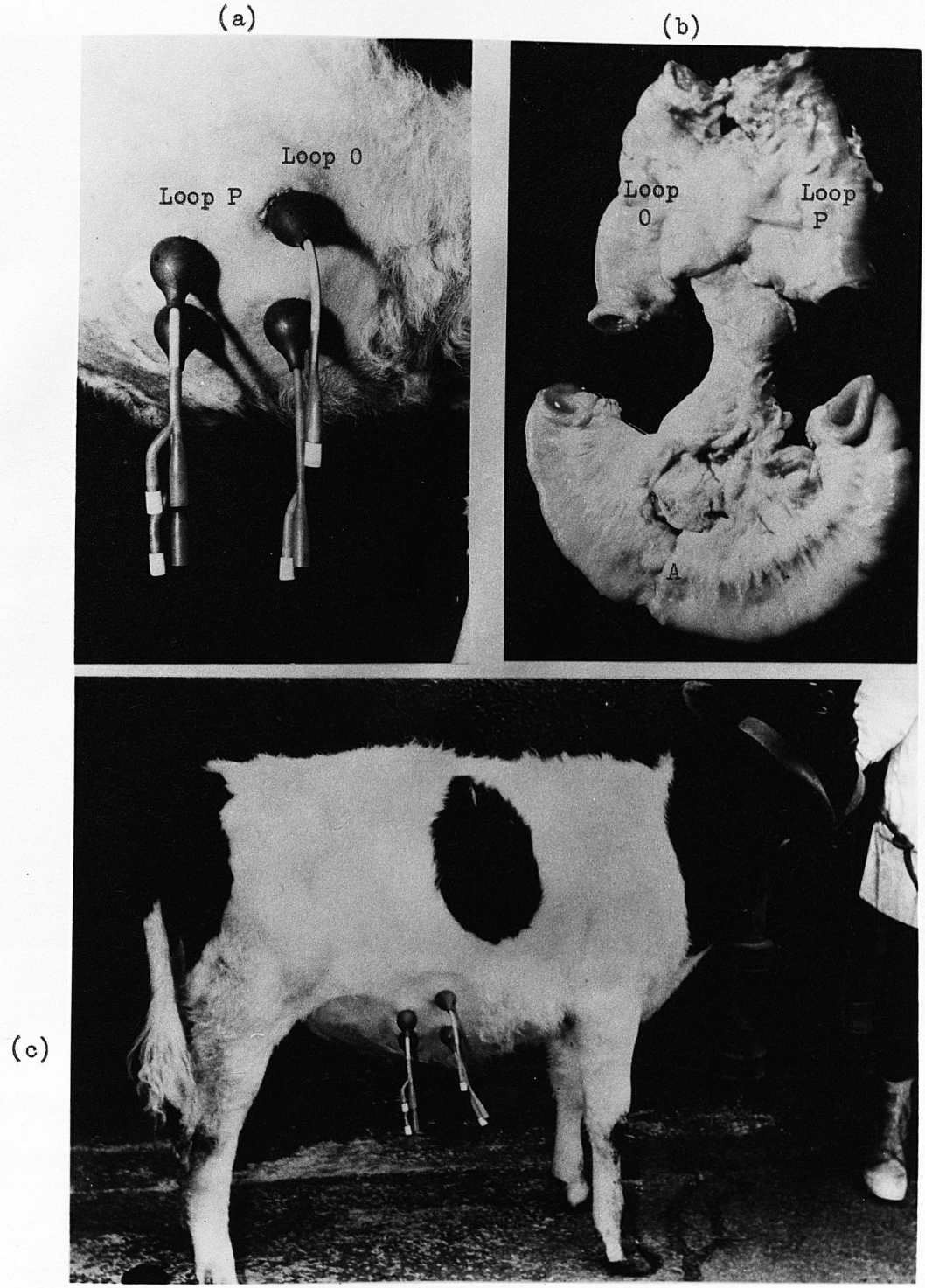


Fig.4 (a) and (c) External appearance of loops O and P with catheters in position. Age of loops 20 weeks.

(b) Post-mortem appearance of loops O and P and neighbouring intestine. The site of the anastamosis is marked A. Age of loops 50 weeks.

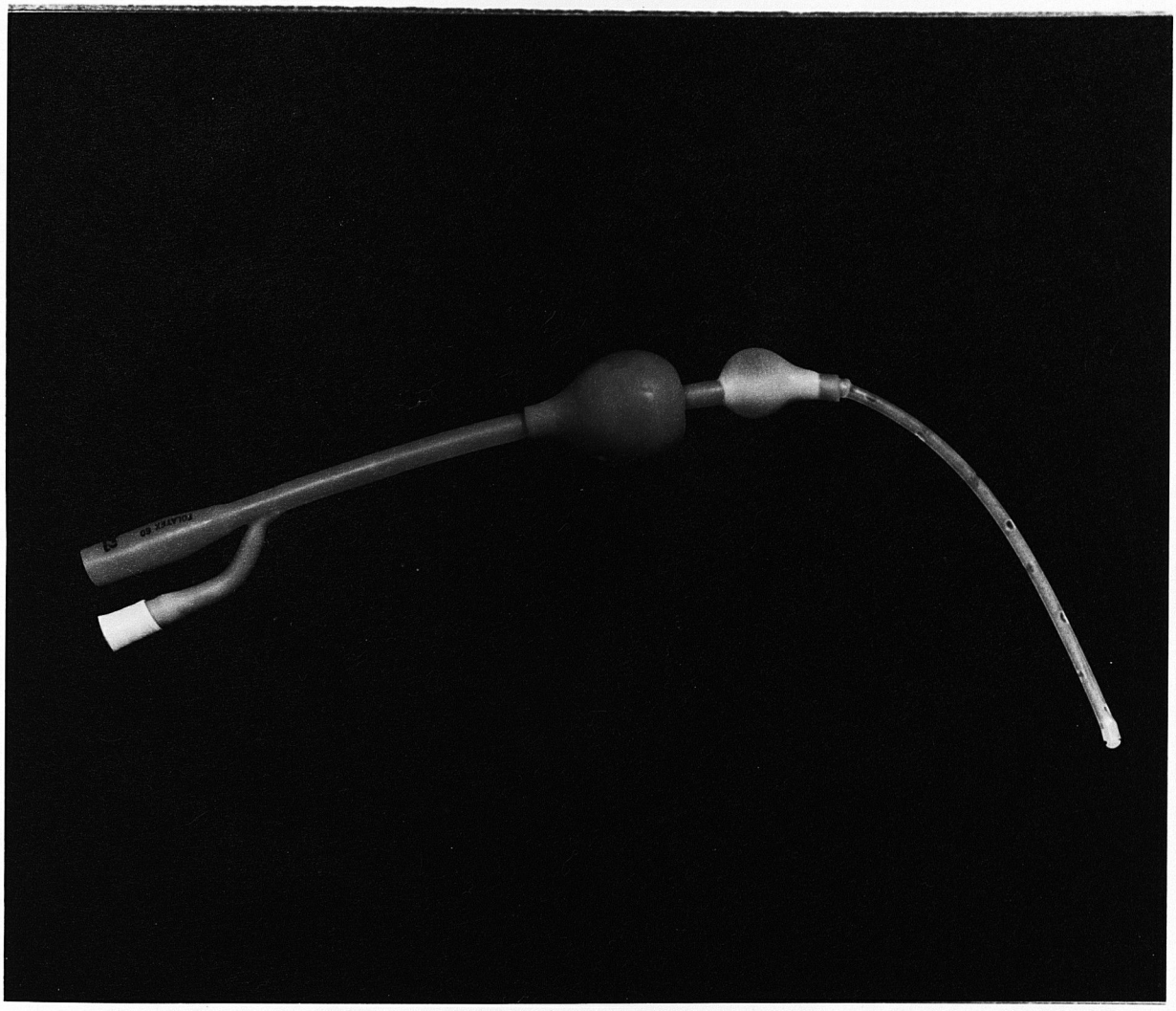


Fig.5 Modified Foley catheter showing the perforated extension and partly inflated balloon.

Closure of stomas.

The ends of the loops could be sealed by Foley catheters (Folatex size 18-22 Fr.female) fitted with 10 cm perforated extensions. The catheters were held at right angles to the abdominal wall by sponge rubber, latex covered cones, or in later experiments by rubber bulbs (Fig. 5). When in use the balloon was inflated with 8 ml of water, and the outer cone or bulb pushed firmly against the abdominal wall. This usually resulted in effective sealing as judged by the absence of visible seepage, but where leakage was seen to occur, the results from that experiment were discarded.

Use of a non-absorbable marker.

To overcome the problem of incomplete drainage of the loops, Polyethylene glycol 4000 (PEG , Koch-Light Ltd) was used as a non-absorbable marker. The suitability of PEG for this purpose was tested by measuring the total recovery of the marker from the fluid drained from the loop after 30 minutes, and also from washings of the loop thereafter.

Samples (0.1ml) for PEG analysis were added to 0.9 ml of distilled water, using a constriction pipette. The method of analysis was the turbidometric method of Hyden (1956) except that turbidity was observed after exactly 60 min (Smith, 1959) at 650 mμ in an E.E.L. spectrophotometer. Determinations were usually carried out in duplicate.

Preparation of enterotoxin.

Organisms (*E.coli* strain B44) were grown on semi-solid agar medium, and the supernatant fluid was separated as described earlier. 30 ml volumes of

culture filtrate were precipitated by 8 volumes of acetone and allowed to stand overnight at -30°C. The precipitate was then dried and redissolved in 30ml of test solution I. This amount of enterotoxin containing material was the standard quantity used in many of the present experiments (1 standard dose). Uninoculated culture medium was treated in an identical manner and used as a control.

Test solution I contained NaCl,(49.56 meq/l); $NaHCO_3$, (26.0 meq/l); KCl,(4.0 meq/l); Glucose,(10g/l except for experiments on loops A & B, where glucose concentration was 1.0 g/l). After addition of acetone extract, this solution was approximately isotonic.

Where no acetone extract was added, a second test solution (II) was used in which the NaCl content was raised to 97.72 meq/l to maintain isotonicity.

All reagents used were A.R.grade.

Analytical Methods.

Sodium and potassium were estimated by flame photometry (E.E.L. flame photometer); bicarbonate by the microdiffusion method (Conway,1962); glucose by the glucose oxidase/dianisidine method (Dahlqvist, 1964); and chloride by the E.E.L. chloridemeter.

Osmolality was determined cryoscopically using a modification of the Hortvet apparatus (Hortvet, 1921) in which the sample size was 20 ml.

Experimental procedure for the study of net transport of fluid and solutes from Thiry-Vella loops.

Before a typical experiment, with the animal standing quietly in a holding-crate, the catheters were placed in position and the balloons inflated with 6-8 ml of water. The cones were moved into position to seal the stomata, and the loops were irrigated with isotonic saline at 37°C. A constant supply of warm saline was provided by a reservoir of saline leading to glass coils in a water bath at 38°C and thence to an outlet.

Irrigation was considered complete when no more mucus and debris appeared in the washings. As much as possible of the washing material was removed with a syringe and the ends of the catheters were then clamped with spring clips.

30ml of control solution (test-solution 1 plus acetone extract of 30ml of uninoculated culture medium filtrate) was placed in the loop using a syringe, and was thoroughly mixed with the residue of the saline. A 0.1 ml sample was then removed for polyethylene glycol analysis to give an estimate of the amount of residual saline in the loop. Exactly 30 minutes were allowed to elapse after addition of the test solution, and the loop was then drained, and samples taken for electrolyte, PEG. and glucose analysis.

The loop was thoroughly washed with saline, and 30ml of the toxin-containing solution (test-solution 1 plus acetone extract of 30ml of enterotoxic culture filtrate) was placed in the loop and treated in the same way as the preceding control. After 30 minutes, the loop was again drained and samples were taken for analysis. Finally, the loops were washed again with saline to remove final traces of toxin. The method of calculation of results is shown in Appendix 1.

Each loop was therefore used as its own control assuming that differences between absorption during the first and second 30 min. periods were a result of enterotoxin activity. This assumption was tested by experiments in which identical solutions (test-solution I plus control extract, or test-solution II alone) were placed in loops during both periods, and absorption of fluid and solutes were thus compared in the absence of toxin.

RESULTS

Control Experiments.

a) Experiments to test whether net absorption differed between consecutive 30 minute periods.

Results of experiments using Test solution II are shown in Table (10). The net absorption of fluid, sodium, potassium and bicarbonate during consecutive 30 minute periods did not differ significantly (t-test on paired samples) in any of the loops examined. Similarly, when solution I plus control extract was used during the two periods, the differences were again not significant. Loops were tested periodically to ensure that double controls were still similar.

b) Suitability of Polyethylene Glycol 4000 as a non-absorbable marker.

In 10 experiments where no leakage had occurred, the mean recovery of Polyethylene glycol was 97% $\pm$ 1.9 (S.E.)

Replicate determinations (10) on a sample of PEG containing 7.5mg/ml. gave a mean and standard error of 7.45 $\pm$ 0.03mg/ml.

Loop	Absorption Period	Net Fluid Absorbed ml/30 min.	Net Sodium Absorbed μeq/30min.	Net Potassium Absorbed μeq/30min.	Net Bicarbonate Absorbed μeq/30min.
G	1st	-3.3±1.0 (6)	-400±130 (6)	-39± 5 (5)	-190±13 (4)
	2nd	-2.6±1.5 (6)	-220±190 (6)	-37± 6 (5)	-260±50 (4)
H	1st	-3.3±1.0 (6)	-400±160 (6)	-53± 6 (5)	-375±60 (5)
	2nd	-2.6±1.4 (6)	-200±210 (6)	-56± 4 (5)	-376±50 (5)
A	1st	+4.7±1.5 (8)	+40 ± 80 (8)	-	-
	2nd	+5.7±1.1 (8)	+60 ±100 (8)		
B	1st	+3.2±1.2 (8)	+960±310 (8)	-	-
	2nd	+2.8±1.4 (8)	+1160±130 (8)		
E	1st	+1.2±0.9 (8)	+340±120 (8)	-	-
	2nd	+1.2±0.8 (8)	+280±110 (8)		
F	1st	-1.5±0.6 (8)	-260±130 (8)	-	-
	2nd	-1.6±0.6 (8)	-320±130 (8)		

Table (10) Results of control experiments using Test sol.II during first and second 30 minute periods. Positive signs indicate net absorption, negative signs net secretion. Figures represent the means and standard errors of the number of observations shown in parenthesis. In no case is there a significant difference between the two absorption periods ($P > 0.05$).

c) Osmolality determinations.

Ten replicate determinations of the osmolality of physiological saline solution (0.95%) gave a mean and standard error of 304 ± 0.62 m.osm/kg.

d) Changes with age of Thiry-Vella loops.

Ability to absorb fluid.

Figs.(6) and (7) show the absorption of fluid from two pair of loops (O & P; I & J) at intervals over thirteen weeks in one case, and forty-eight weeks in the other. These results show that the loops retained their ability to absorb fluid over considerable periods, and did not show substantial deterioration with age. Both of these loops were situated in the distal ileum about 2m from the ileocaecal valve.

Ability of loops to continue to respond to enterotoxin.

Figs.(8) and (9) show the response to enterotoxin of the four loops described in the previous section. Each point represents the difference between the absorption of fluid during the control period and subsequent test period when the loop contained acetone extract from 30ml of culture filtrate. There appeared to be little or no loss of the ability to respond to enterotoxin with increasing age of the calf and of the Thiry-Vella loop.

Histological changes in Thiry-Vella loops.

After the animals were killed, tissue was taken from each loop and preserved in formal-saline for subsequent examination. Tissue was also removed from a section of intestine within 20cm of the point at which the gut had been anastamosed.

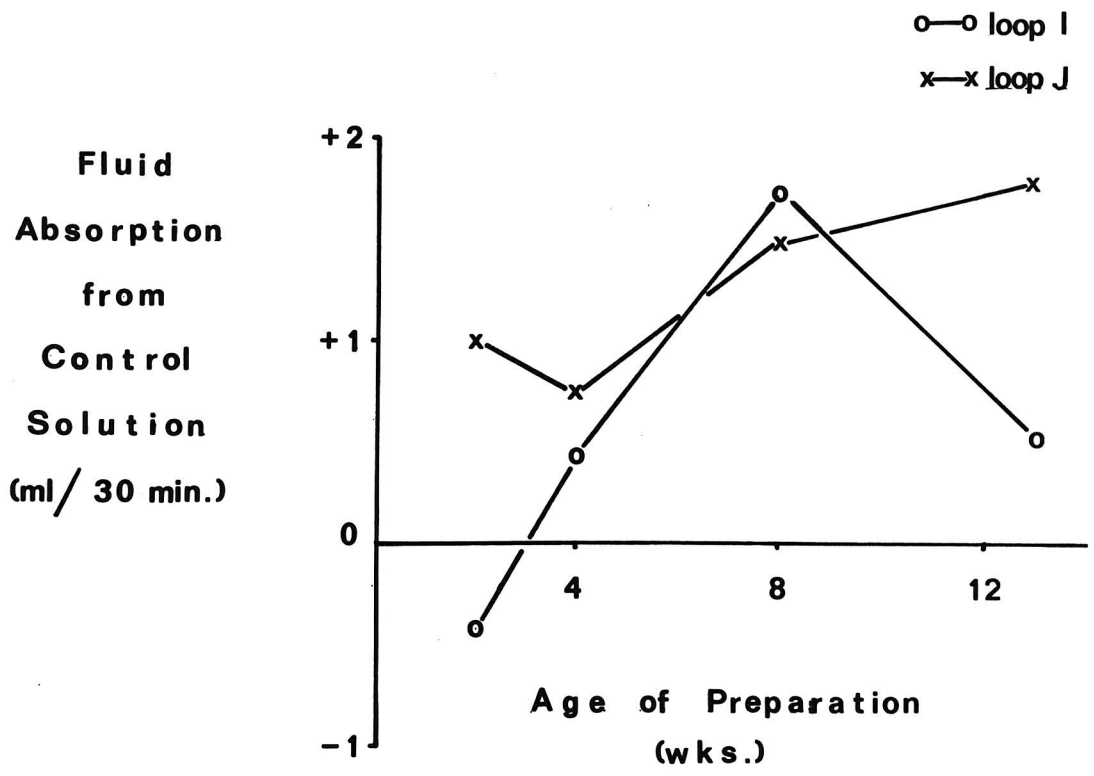


Fig.6 The effect of age of loops I and J on net fluid absorption.

Net movement of fluid (ml per 30 min) from control extract dissolved in test-solution I in loops I and J tested at intervals over a period of 12 weeks. Positive signs indicate net absorption, negative signs show net secretion. Each point represents the result of a single experiment.

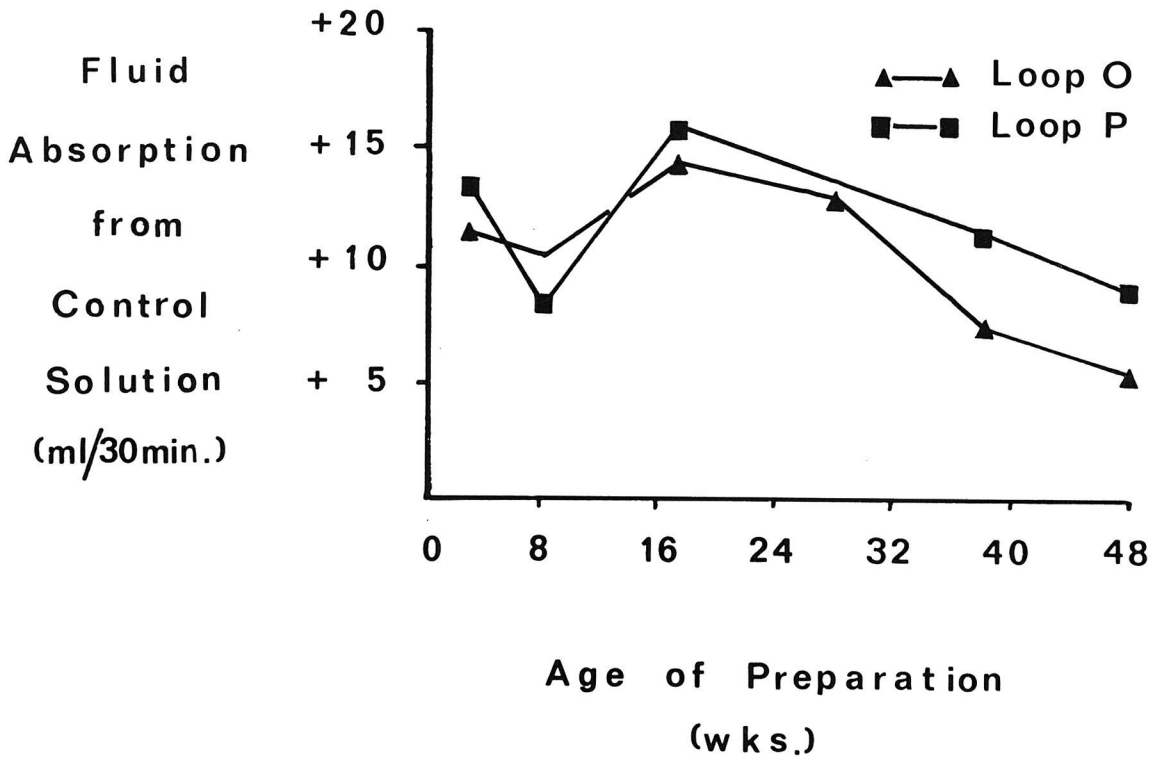


Fig.7 The effect of age of loops O and P on net fluid absorption.

Net movement of fluid from control solution in loops O and P tested at intervals over a period of 46 weeks. Notation is as in Fig. 6.

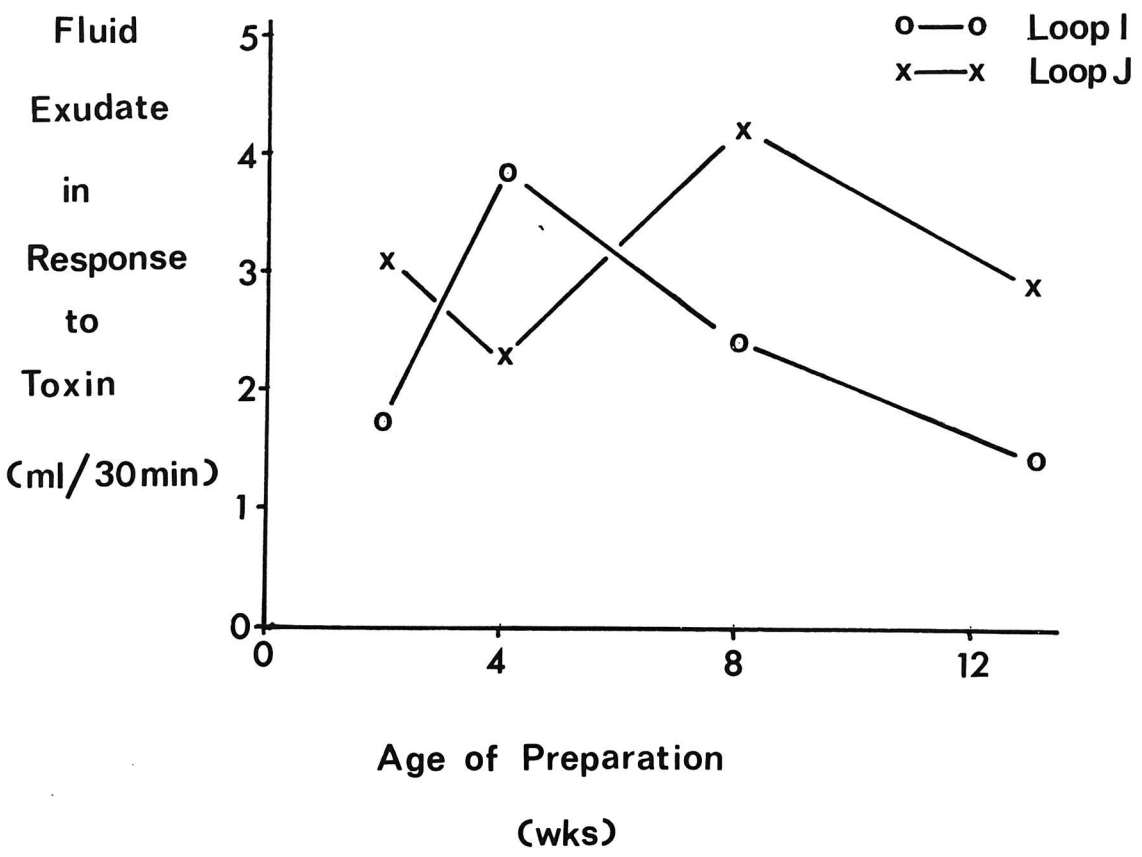


Fig.8 The effect of age of loops I and J on response to enterotoxin.

Fluid exudate in response to one standard dose of enterotoxic extract in loops I and J tested at intervals over a period of 12 weeks. Each point represents the difference between the absorption of fluid from control and enterotoxic solutions during consecutive 30 minute periods.

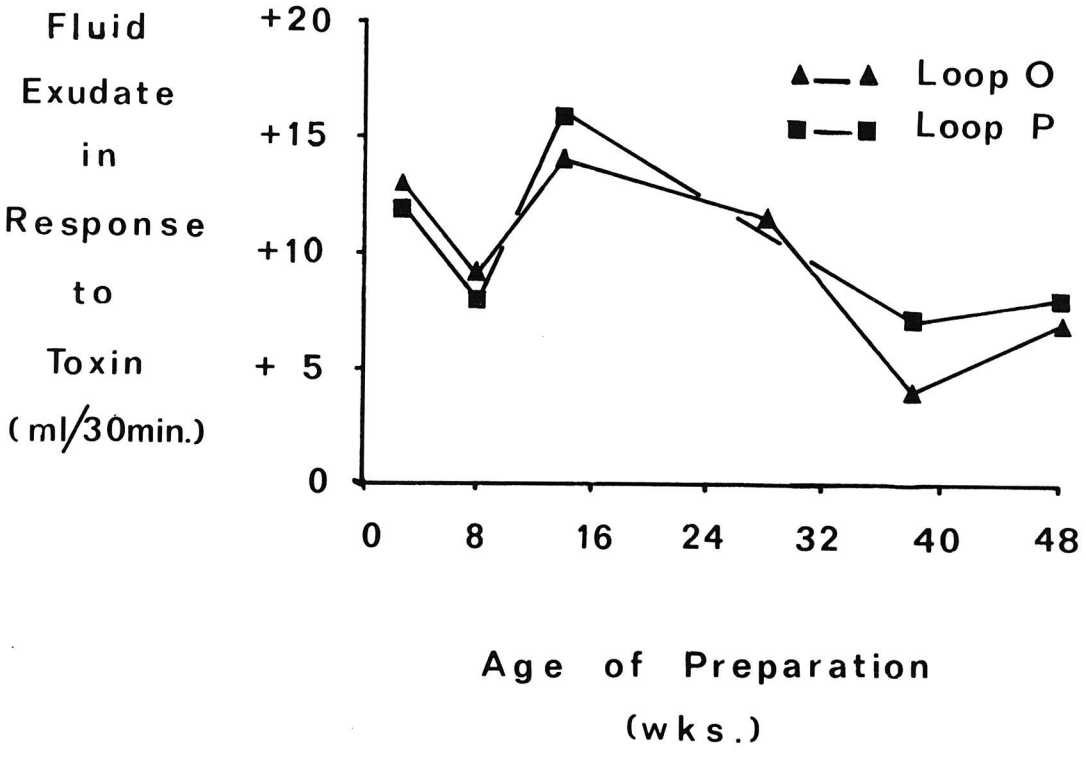


Fig.9 The effect of age of loops O and P on response to enterotoxin.

Fluid exudate in response to one standard dose of enterotoxic extract in loops O and P tested at intervals over a period of 46 weeks. Notation is as in Fig.8.

Histological examination showed that the mucous membrane of the Thiry-Vella loops was thinner and less folded than that of neighbouring intestine. The control material showed more desquamation of mucosal epithelium than did the section of loop mucosa, despite the identical manner in which the samples were taken. Fig (10) shows sections taken from a loop placed in distal ileum and from neighbouring intestines. The villi in such loops were not materially blunted in comparison with control sections. In loops in the upper intestine, however, blunting of villi could sometimes be seen.

In some cases (e.g.Fig.10) control sections showed lymphoid infiltration of the submucosa in comparison with the sections from Thiry-Vella loops.

The effect of enterotoxin on net fluid, glucose and electrolyte movement - Results.

a) Effect on net fluid movement.

Fig.(11) shows the effect of enterotoxin net fluid movement in eight Thiry-Vella loops (4 calves). The amount of enterotoxin used in each case was that derived from 30ml of culture fluid (E.coli strain B44). In all eight loops the enterotoxin caused a significant shift towards net secretion in comparison with the preceding control period.

b) Effect on net sodium movement.

Fig.(12) shows the effect of enterotoxin on net sodium absorption from eight loops. Conditions were as above, and the effect was in each case to cause a net movement of sodium towards the gut lumen.

Fig.10 Histological appearance of mucosa from loop I (lower) in comparison with that of neighbouring intestine (upper). Magnification 17x.

c) Effect on net chloride and bicarbonate movement.

Fig.(13) shows the effect of enterotoxin on net chloride and bicarbonate movement in four loops. In each case the presence of enterotoxin has caused a significant movement of ions towards the gut lumen.

d) Effect on potassium movement.

Table (11) shows the effect of enterotoxin on net movement of potassium. In the dose used (acetone extract from 30ml. of culture filtrate) there was a significant effect in only two out of eight loops examined. In these two, the effect was to increase net movement of potassium into the loops.

e) Effect on glucose movement.

Table (11) shows the effect of enterotoxin on net movement of glucose. In the dose used, there was no significant effect on glucose movement in any of the loops examined.

f) Osmolality of enterotoxic and control solutions.

Osmolality of control solution (test solution I plus extract of uninoculated culture medium) was 322 $\pm$ 6 m.osm/kg. while osmolality of the enterotoxic solution was 328 $\pm$ 11 m.osm/kg. These do not differ significantly. ($p > 0.05$).

Loop	E	-	Upper jejunum
"	F	-	" "
"	G	-	" "
"	H	-	" "
"	A	-	Lower ileum
"	B	-	" "
"	I	-	" "
"	J	-	" "
"	O	-	" "
"	P	-	" "
"	Q	-	" "
"	R	-	" "
"	U	-	" "
"	V	-	" "

Table 10(b) The position of the Thiry-Vella loops in the small intestine.

Table 10(b) shows the site of the Thiry-Vella loops.

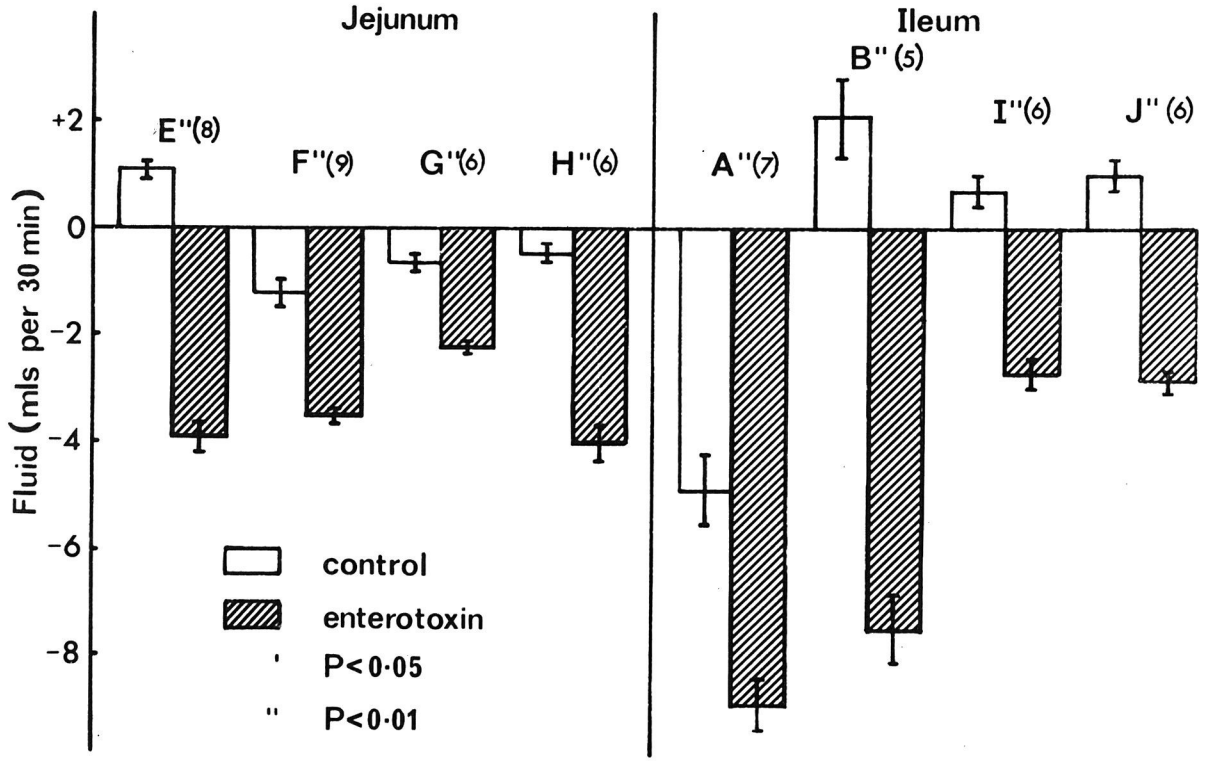


Fig.11 The effect of enterotoxin on net movement of fluid from eight Thiry-Vella loops.

Loops E,F,G, and H (2 animals) were situated in the upper jejunum, loops A,B,I and J (2 animals) were situated in the lower ileum. Each column represents the mean fluid movement in the presence of control extract (open columns), or enterotoxic extract (1 standard dose, hatched columns) during consecutive 30 minute periods. The number of experiments in each case is shown in parenthesis, and the standard errors are shown as vertical bars.

Positive signs indicate net absorption, negative signs show net secretion. Differences were compared using the paired t-test.

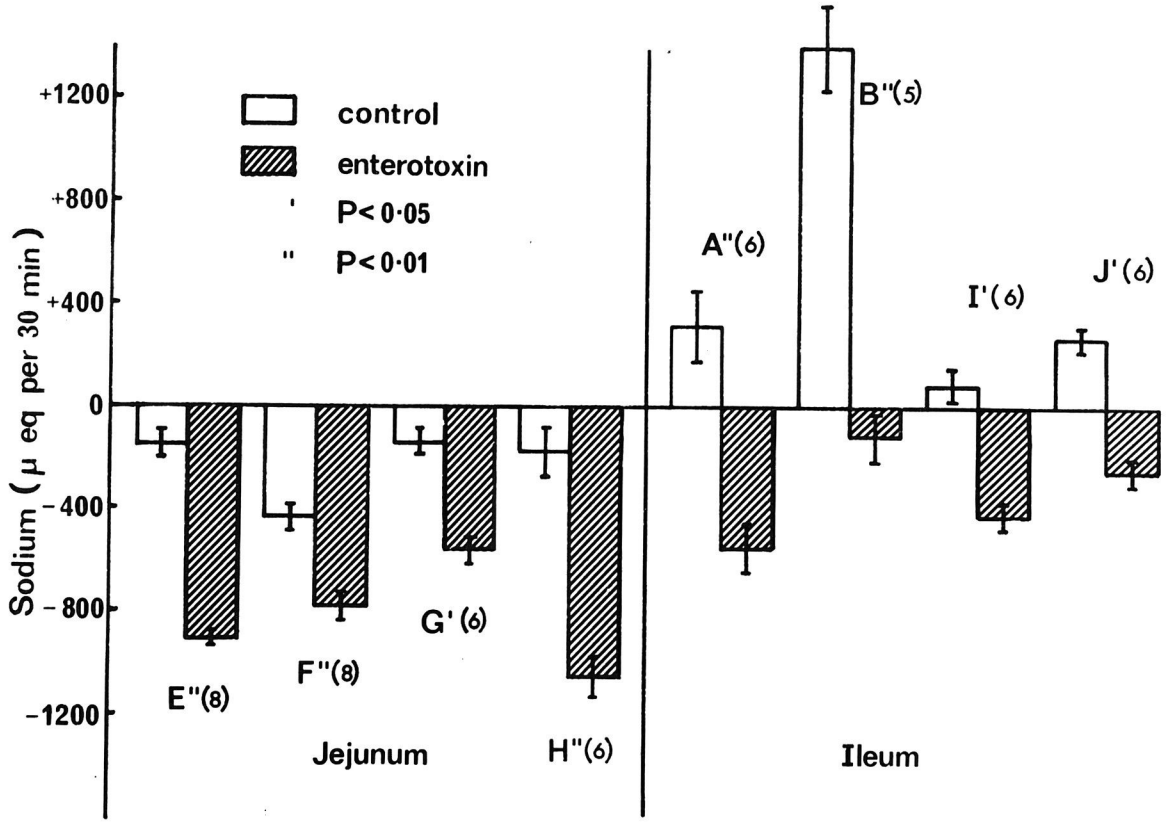


Fig.12 Net movement of sodium from eight Thiry-Vella loops in the presence of control and enterotoxic material. Loops and notation as in Fig.11.

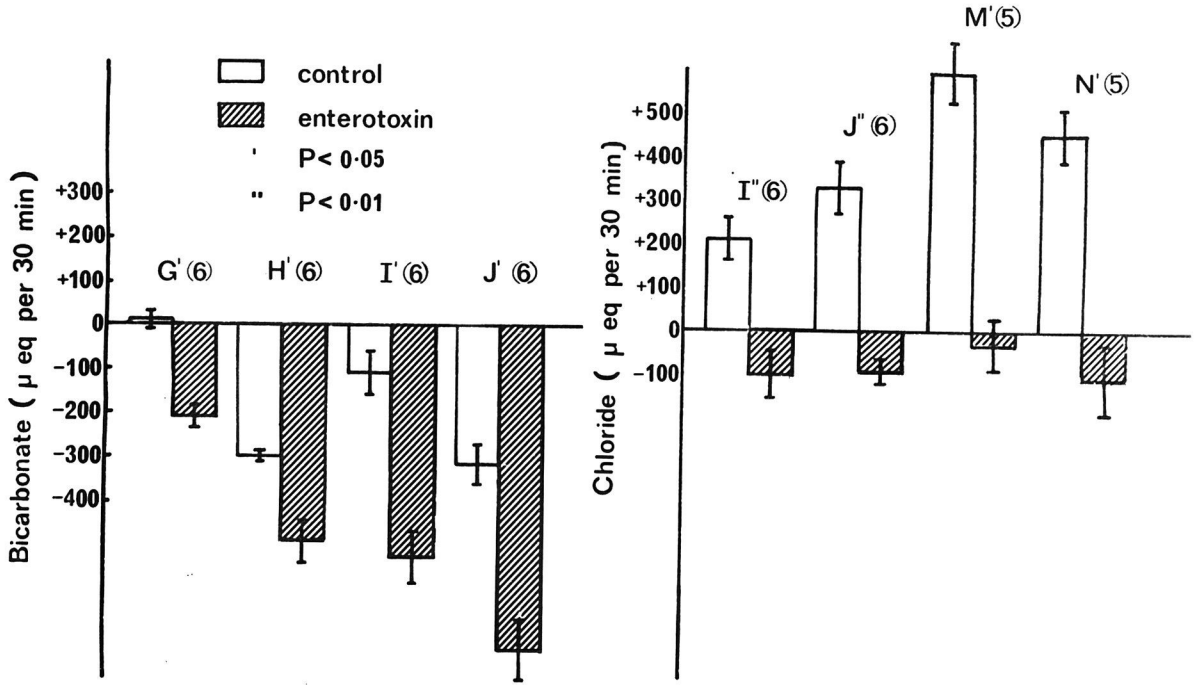


Fig.13 Net movement of bicarbonate (loops G,H,I and J) and chloride (loops I,J,M and N) in the presence of control and enterotoxic extracts. The notation is as in Fig. 11.

		Loop E	Loop F	Loop G	Loop H	Loop A	Loop B	Loop I	Loop J
POTASSIUM (μeq/30min)	CONTROL	-54±38	-30±16 *	-16±12	-36±3	-226±30	-141±34 *	-71±8	-103±13
	TOXIN	-71±15	-73±16	-28± 6	-48±4	-229±28	-234±34	-86±8	-111±12
GLUCOSE (mg/30min)	CONTROL	+152±14	+78±10	+27± 5	+62±11	+24± 4	+27 ± 1	+26±6	+25 ± 4
	TOXIN	+145±17	+76±10	+22± 4	+48±12	+19± 2	+29 ± 4	+13±6	+31 ± 1

Table (11) The effect of enterotoxin on potassium and glucose absorption. Figures represent the means of at least five observations ± SE. Positive signs indicate net absorption, negative signs indicate net secretion. The asterisk (*) shows a significant difference between the corresponding enterotoxin and control results ($p < 0.05$, paired t-test).

DISCUSSION

Thiry-Vella loops have been shown to respond rapidly to the presence of enterotoxin in the intestinal lumen, and appear to have several advantages over methods previously used for the study of E.coli enterotoxin activity:-

1. They allow replication of experiments within the same animal.

2. They allow easy access and sampling of luminal fluid to monitor changes in fluid and electrolyte movement during enterotoxin activity.

3. They are economical in the use of experimental animals.

The use of consecutive absorption periods for the study of enterotoxin activity has been shown to be justified, and polyethylene glycol 4000 has been shown to be a suitable non-absorbable marker.

Thiry-Vella loops have been shown to retain their ability to absorb fluid, and also to respond to enterotoxin activity, over a long period. On the other hand histological changes in the mucosa of loops in comparison with neighbouring normal intestine suggested that the lack of luminal nutrition may have produced some 'unphysiological' effects. However, since each experiment included a control period, it seemed likely that any abnormalities might be unimportant in the study of changes caused by enterotoxin activity.

The effect of enterotoxin activity on net movement of fluid, sodium, chloride and bicarbonate was to cause a significant shift towards secretion. Potassium was inconsistently affected, and glucose disappearance was unaffected.

These changes correspond reasonably closely to the water and electrolyte losses which occur in diarrhoea of calves. Scouring calves have been shown by several authors to lose large amounts of fluid in their faeces (Blaxter and Wood, 1953; Fayet, 1968a). There is also a marked fall in serum sodium

(McSherry and Grinyer, 1954; Roy, Shillam, Hawkins, Lang and Ingram, 1959; Masek, 1968; Fayet, 1968b) and in serum bicarbonate (McSherry and Grinyer, 1954; Fayet, 1968b).

These changes might be predicted from the present experiments, as might the rise in serum potassium which has been shown to occur in scouring calves (Roy et al., 1959; Masek, 1968; Fayet, 1968b), since loss of fluid with relatively little loss of potassium would raise the serum potassium concentration.

Serum chloride, however has also been shown to rise during diarrhoea (Masek, 1968; Fayet, 1968b) and this would not have been predicted from the present results, since chloride loss was increased by the presence of enterotoxin.

The effects on the net fluid, sodium, bicarbonate and chloride transfer resemble the effect of Vibrio cholerae enterotoxin in the rabbit (Norris, Curran and Schultz, 1969) and in the dog (Swallow, Code and Freter, 1968). These authors found that potassium movement was also significantly affected, (although less dramatically than the other parameters studied), but Love (1969) found little change in potassium movement in rabbit intestine in response to V.cholerae infection.

The lack of any effect on glucose absorption is similar to results obtained by other workers using Vibrio cholerae enterotoxin in the dog (Carpenter, Sack, Feeley and Steenberg, 1968) and in the rabbit (Serebro, Bayless, Hendrix, Iber and McGonagle, 1968.) This may indicate that neither agent has a generalised toxicity for mucosal cells, that the reserve capacity for glucose absorption is considerable, or that the cells affected by entero-

toxin activity are spacially separated from those involved in glucose absorption (Hendrix and Banwell, 1969).

The effect of E.coli enterotoxin was similar in loops of either upper or lower intestine. This differs from the findings of Smith and Halls (1967a,b) who found that ligated loops of upper intestine were more sensitive to dilatation than loops of lower intestine. They found the last 3m, the site of the ileal loops in the present experiments, to be unreactive. The difference may result from differences in absorptive behaviour in upper and lower small intestine since in the present experiments, 3 out of 4 loops placed in the upper jejunum showed net secretion of fluid, while 11 out of 12 loops in the lower ileum showed net absorption of fluid. Similar differences have been demonstrated in dog intestine (Carpenter et al., 1968.) It is possible therefore, that ligated loops of distal ileum may show less dilatation in response to enterotoxin as a result of their greater absorptive capacity which would have to be overcome before dilatation could occur.

The fact that calf intestine continues to respond to enterotoxin for some twelve months is in contrast with the report that oral administration of enterotoxic organisms can produce diarrhoea in calves only if carried out during the first twenty hours of life (Smith and Halls, 1967a). This supports the suggestion of Smith and Halls, (1967a), that for a strain to be enteropathogenic, it must have the ability to proliferate in the first part of the intestine as well as the ability to produce enterotoxin. This proliferation in the upper intestine may be associated with the ability of organisms to attach themselves to the intestinal mucous membrane (Arbuckle, 1970).

Moon and Whipp (1970) using ligated loops in pigs, found that while some strains of E.coli caused dilatation of loops when live cultures were injected into loops of pigs of up to twelve months old, the response to other strains was lost when tested in pigs over nine weeks of age. They suggested a number of explanations for this, but since they used living cultures rather than cell free material, the situation is complicated by factors which affect the multiplication of microorganisms.

2. THE EFFECT OF ENTEROTOXIC EXTRACT ON UNIDIRECTIONAL FLUXES OF FLUID AND SODIUM.

INTRODUCTION.

The effect of enterotoxin on net fluid and sodium movement could have resulted from an increase in the movement of material from blood to lumen (exsorption) or a decrease in movement from lumen to blood (insorption) or both. The terms 'insorption' and 'exsorption' are those used by Code, (1960).

It seemed that if the effect of the enterotoxin could be defined in terms of changes in the unidirectional fluxes of fluid and sodium, then this might be of value in the understanding of its mode of action.

MATERIALS AND METHODS.

Deuterium oxide (1%w/v) and ^{22}sodium (2μc/l) were used as isotopic tracers to assess the unidirectional movements of sodium and water across the small intestine.

Experimental procedure.

The experimental procedure resembled that described in the previous section in that each loop was used as its own control in paired experiments. The time intervals differed, however:-

a) In the first experiments carried out with isotopic tracers, the isotopes were added to test-solution II (page 52) and absorption was measured during 10 minute periods following 45 minutes exposure to control or enterotoxic solutions respectively. The amount of toxin used was one standard

dose (i.e. that derived from 30 ml. of culture filtrate).

b) In later experiments, the isotopes were added to test-solution I (page 52) together with either control or enterotoxic extract, 1 standard dose in each case. Unidirectional fluxes were then measured during consecutive 10 minute periods in the presence of control and enterotoxic material respectively.

Control experiments were carried out by measuring fluxes to and from identical solutions during both periods.

The method of calculation of the results is shown in Appendix 2. The calculations involve certain assumptions:-

1. That the labelled and unlabelled material leave the intestinal lumen in the proportion they bear to one another in the lumen. This seemed a particularly questionable assumption in the case of deuterium oxide, in view of the molecular weight difference between D_2O (or HDO) and H_2O, and also in view of the equivocal nature of the evidence from the literature (Appendix 3). Experiments were therefore carried out on rat intestine in vitro to determine whether deuterium oxide transport in this preparation was indistinguishable from that of water. These experiments are described in Appendix 3, and the results show the presence of a significant isotope effect of deuterium oxide. It is suggested, however, that this is unlikely to greatly affect the results of experiments to determine the effect of enterotoxin on unidirectional fluid fluxes.

2. That on absorption, the labelled material is rapidly removed to the circulation and does not re-enter the lumen or accumulate within the mucosa. The absence of re-entry was partly verified by measuring the radio-

activity entering a non-radioactive solution placed in a loop for a ten minute period immediately after an experiment. Accumulation of isotope within the mucosa could have been examined by killing an animal immediately after an experiment. This was impracticable.

3. That the rise in the concentration of isotope in the serum was small during the experiment. This was verified by estimating ^{22}Na and deuterium oxide in serum samples before and after an experiment.

Analytical methods.

^{22}Sodium was estimated by scintillation counting of gamma radiation in a NaI well-type scintillation counter (Panax). A minimum of 8,000 counts (net) were recorded for each sample.

Deuterium oxide was estimated by infra-red absorbance at 3.98 μ (Turner, Neely and Hardy, 1960) using a Perkin Elmer Model 257 double beam spectrometer. Samples were first distilled to dryness in an all glass micro-distillation apparatus, and the distillate placed in spectrometer cells with calcium fluoride windows (Unipak cells, Ross Scientific Co Ltd.) The absorbance was compared with that of a series of standard preparations. A fresh calibration line was established each time the method was used.

RESULTS.

Control experiments.

It was found that negligible amounts of radioactivity re-entered the Thiry-Vella loops during the period immediately following one during which the label had been present in the loop. It was also found that the increase in the serum concentrations of the isotopes was negligible during the period of an experiment.

The experiments using rat intestine *in vitro* (Appendix 3) showed that in this preparation there was a significant isotope effect of deuterium oxide.

Table (12) shows the mean fluxes during two consecutive ten-minute periods with control extract (from uninoculated culture medium) in loops O and P during both periods. There is no significant difference between any of the respective pairs of fluxes. This was confirmed in other loops.

Experiments using enterotoxin.

The experiments in which the fluxes were measured after a 45 minute exposure to enterotoxic extract were found to give less clear results than those in which the fluxes were measured in the presence of enterotoxic material. The results to be described were those obtained using the latter method.

a) Effect of enterotoxin on unidirectional fluxes of fluid.

The effect of enterotoxic extract on unidirectional fluxes of fluid in 6 Thiry-Vella loops (O,P,Q,R,U and V) is shown in Figs (14 and 15a). These results were obtained from 10-minute experimental periods in the presence of enterotoxin. No allowance was made for possible isotopic effect of deuterium.

		Test Period	LOOP O	LOOP P
FLUID (ml /10min)	INSORPTION	UCM 1	26.1 ± 2.2	20.6 ± 0.7
		UCM 2	29.1 ± 2.1	19.2 ± 2.2
	EXSORPTION	UCM 1	24.7 ± 2.2	18.6 ± 0.7
		UCM 2	24.2 ± 3.2	19.9 ± 1.0
SODIUM (μeq /10min)	INSORPTION	UCM 1	865 ± 25	890 ± 115
		UCM 2	960 ± 61	840 ± 72
	EXSORPTION	UCM 1	502 ± 110	484 ± 84
		UCM 2	600 ± 110	410 ± 91

Table (12) Control results for unidirectional flux determinations.

Unidirectional fluxes of fluid and sodium in loops O and P during two consecutive 10 min. periods with control extract (UCM) in the loops during both periods. Figures represent the means ± SE for four experiments.

In no case is the flux during the second period significantly different from that during the first ($P > 0.05$ paired t-test).

When the results from each loop were considered individually, no significant effect of enterotoxin on insorption of fluid in 5 out of 6 loops was seen. The sixth loop (loop V) showed significantly increased insorption. However, by combining the results for fluid insorption in all six loops a significant overall increase in insorption in the presence of enterotoxin ($p < 0.05$, paired t-test)was shown.

The effect of enterotoxin on fluid exsorption (Figs 14 and 15a) was to cause a significant increase in all 6 loops.

Effect of enterotoxin on unidirectional fluxes of sodium.

The effect of enterotoxin on unidirectional sodium fluxes in 6 Thiry-Vella loops (U,V,O,P,Q and R) is shown in Figs (15b and 16).

Sodium insorption was significantly decreased in 5 of the 6 loops. Sodium exsorption was significantly increased in 2 of the loops.

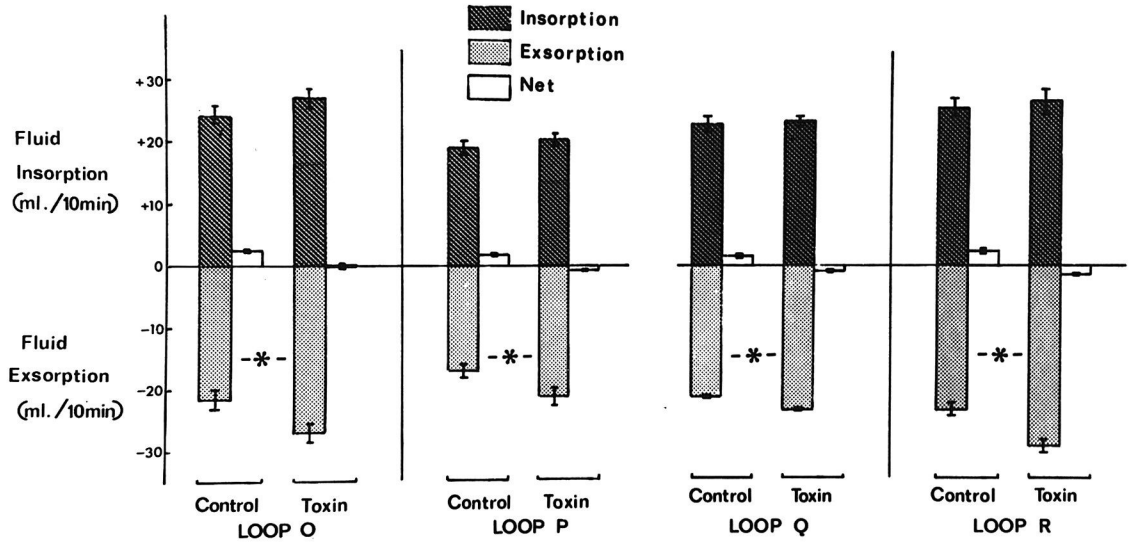


Fig.14 Unidirectional and net fluid fluxes in four Thiry-Vella loops (O,P,Q and R) in the presence of control and enterotoxic solutions during consecutive 10 minute periods. The enterotoxic solution contained 1 standard dose.

Each column represents the mean of at least 5 experiments, and standard errors are shown by vertical bars. Significant differences between unidirectional fluxes in the presence of control and enterotoxic solutions are shown by asterisks (* $P < 0.05$; paired t-test). Differences between net movements (not indicated by asterisks) were significant in each case ($P < 0.05$).

(a)

(b)

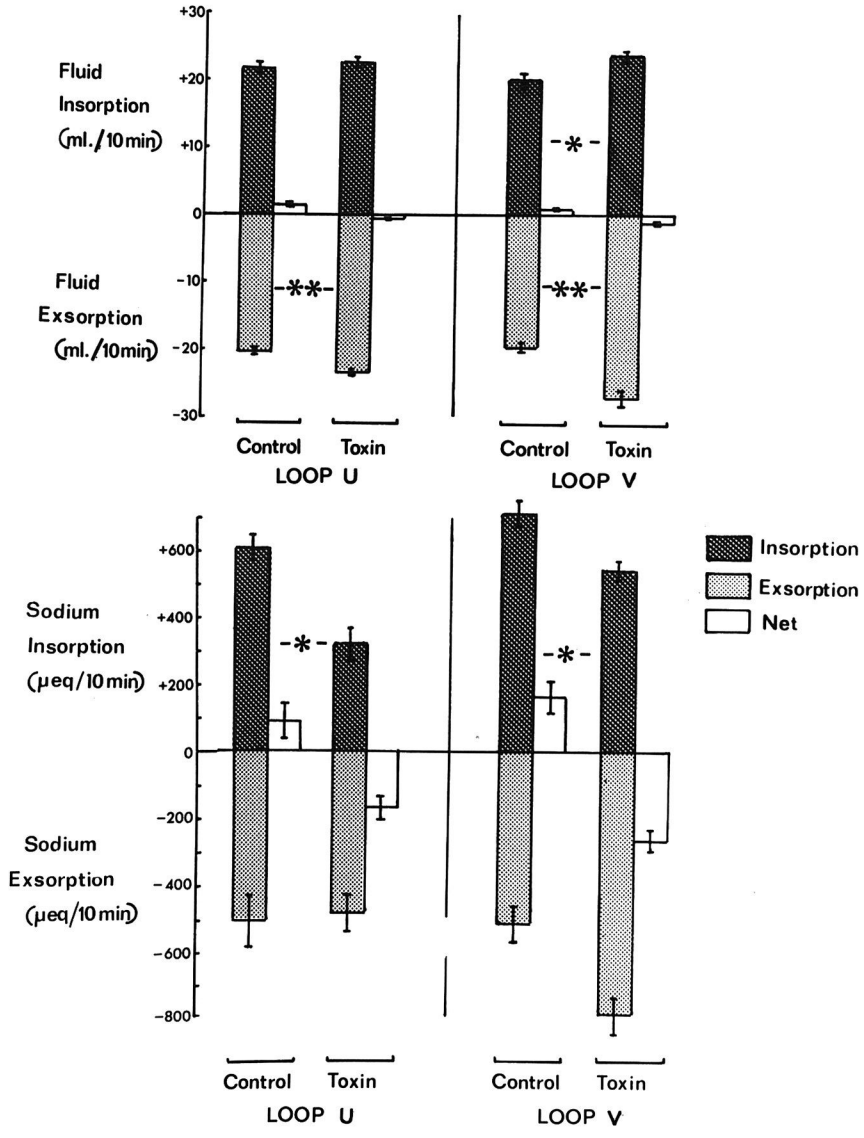


Fig.15 (a) Unidirectional and net fluid fluxes in two Thiry-Vella loops (U and V). Notation is as in Fig.14. (* $P<0.05$; ** $P<0.01$ for unidirectional fluxes) Differences between net movements were significant in each case ($P < 0.05$).

(b) Unidirectional and net sodium fluxes in loops U and V. Notation is as in Fig.14. Differences between net movements were significant in each case. ($P < 0.05$)

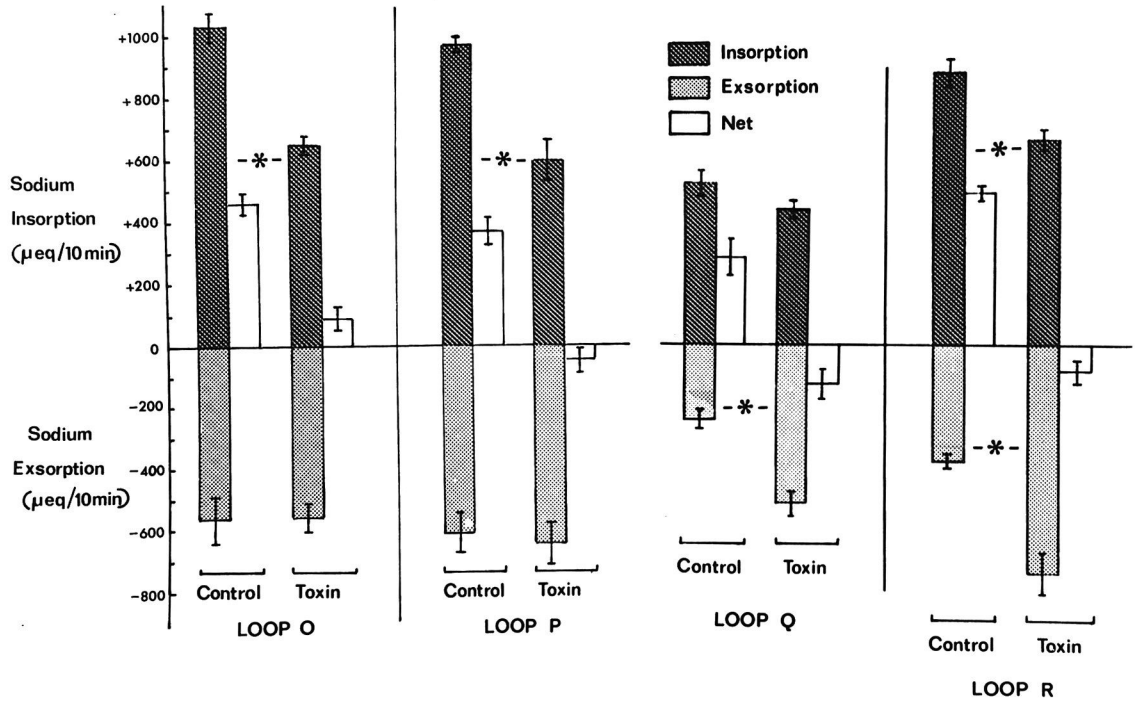


Fig.16 Unidirectional and net sodium fluxes in loops O,P,Q and R. Notation is as in Fig. 14. Differences between net movements were significant in each case.

DISCUSSION.

Unidirectional movement of fluid was affected by enterotoxin (Figs 14 and 15a). Exsorption was increased significantly in all six loops examined. Insorption was significantly increased in one loop, but when the results from all six loops were combined, it was found that there was also a significant overall increase in fluid insorption ($p < 0.05$, t-test). However, the increased exsorption was in each case greater than the increased insorption, so that the consistent net effect was to cause a shift towards secretion.

Sodium movement was affected in a less uniform manner. Sodium insorption was significantly decreased in 5 of the 6 loops, while in the sixth, the reduction was not significant. Sodium exsorption was increased significantly in 2 of the 6 loops. The net effect therefore, in contrast with that on fluid movement, appeared to be principally a result of decreased insorption, but in two of the six loops the net effect was also in part the result of increased exsorption.

No work has so far been done to indicate whether the changes seen in Thiry-Vella loops in response to enterotoxin resemble those in the intact animal during diarrhoea. A brief communication of Whitten and Phillips (1970) has described experiments carried out *in vitro* using segments of intestine taken from normal and scouring calves. They arranged the segments in an apparatus in which mucosal and serosal surfaces could be perfused independently with oxygenated salt solution. Isotopic labels (tritiated water and ^{22}Na) were placed in the serosal fluid to follow unidirectional movements of water and sodium. Although the results described lack detail it appears that these authors found that in material from healthy animals there was no net movement

of electrolyte from mucosal to serosal surfaces. In the material from scouring animals significant mucosal gains and losses occurred for sodium and potassium, while chloride stayed in equilibrium. The appearance of isotopic sodium on the mucosal surface was the same for diarrhoeic and control intestine (presumably indicating that sodium exsorption was similar in the two groups). The specific activity of tritiated water on the mucosal surface rose more in the diarrhoeic material than in the controls (presumably indicating that fluid exsorption was greater in material from diarrhoeic animals).

These results agree in general with the present findings for enterotoxic effects in Thiry-Vella loops. However, a particular problem with an in vitro method such as used in the experiments of Whitten and Phillips (1970) is the maintenance of adequate oxygenation of such a thick tissue as calf intestine. The fact that the control material did not appear to transport sodium suggests that it may not have been viable.

Unidirectional movements of fluid and sodium in human patients with diarrhoea associated with E.coli were studied by Banwell, Gorbach, Mitra and Pierce and reported briefly (1970). They used ^{24}Na and tritiated water as isotopic labels and found that both insorption and exsorption of sodium and fluid were raised by 2-3 fold in patients with diarrhoea. These values fell to normal during convalescence as the net mucosal gain was reversed. These findings agree with the present results for fluid movement, where both unidirectional fluxes tend to be increased by enterotoxin activity. They do not agree, however, with the results for sodium movement.

Comparison between E.coli enterotoxin effects and those of cholera enterotoxin.

Banwell et al., (1970) noted the similarity between fluid and electrolyte changes in the E.coli diarrhoea in man and those of cholera.

The effect of cholera enterotoxin on unidirectional sodium fluxes in Thiry-Vella loops in dogs was examined by Swallow et al., (1968). They found that either insorption, exsorption, or both could be affected in order to produce the net mucosal accumulation of sodium. This agrees with the present findings for the effect of E.coli enterotoxin on sodium movement, but not on fluid. Other workers, however, have found in the dog (Iber, McGonagle, Serebro, Leubbers, Bayless and Hendrix, 1968) and the rabbit (Love, 1969; Grayer, Serebro, Iber and Hendrix, 1970) that cholera enterotoxin affects sodium exsorption but not insorption. This agrees more closely with the present findings for fluid fluxes in response to E.coli enterotoxin. Unfortunately, of the above quoted work on cholera enterotoxin, only Swallow et al. (1968) included a study of unidirectional fluid movement (using deuterium oxide as a label). They found inconsistent effects on fluid movement as they had for sodium, with either insorption or exsorption being affected to produce a net change in fluid movement.

Possible mechanisms involved in the effects of enterotoxin on unidirectional fluxes.

The results of enterotoxin activity on unidirectional fluxes of fluid and sodium, present the paradox that the net effect on fluid movement is principally the result of increased exsorption, while the net effect on sodium is principally the result of decreased insorption.

The results obtained for fluid movement might be explained in part on the basis of hypersecretion produced by E.coli enterotoxin. This concept has been used to explain the pathogenesis of cholera (Hendrix and Banwell,1969), where it was suggested that cholera enterotoxin causes secretion in the crypt region of the intestine, but has no effect on the absorptive region of the villus. This explained experiments where cholera enterotoxin caused little change in sodium insorption and glucose absorption, both of which were associated with the villus region. The present results for fluid movement are in partial agreement with such a hypothesis, since the effect of coli enterotoxin is mainly on exsorption of fluid. Moreover glucose absorption is also unaffected. However, a small but significant increase in fluid insorption was seen, which is not in keeping with an effect simply on secretion. The effects on sodium are also incompatable with this explanation alone, and are discussed later.

An alternative explanation of the effects on fluid movement may lie in an effect of enterotoxin on mucosal permeability. If this occurred, it might be expected to cause an increase in the fluid fluxes in both directions, but the hydrostatic effect of blood pressure would increase fluid exsorption more than fluid insorption. The other factor affecting fluid fluxes in the presence of increased pore size would be the osmolality of the lumenal contents, but in this case both control and enterotoxic solutions were approximately isotonic. The observed effects on fluid fluxes fit quite well with the above predictions. However, increases in fluid fluxes resulting from increased pore size would seem likely to be accompanied by similar effects on sodium movement. This was not found to be the case.

The results for sodium movement are difficult to interpret, particularly in relation to fluid movement. It is widely accepted that water movement is passive and secondary to the movement of solutes, particularly NaCl (Schultz and Curran, 1968). If this is so, then it is difficult to explain the findings for loops O,P,R,U and V, where a significant decrease in sodium insorption is associated with an overall significant increase in corresponding fluid insorption.

When the net movement of sodium and fluid from Thiry-Vella loops was compared (Fig.17) it was found that there was a significant regression of net sodium on net fluid movement. This agrees with the concept of passive movement of water following active transport of solute (Schultz and Curran, 1968).

However, when the unidirectional fluxes of sodium and water were compared in six loops (Fig.18) no relationship could be demonstrated between the two. This, together with the findings that enterotoxin appears to produce opposite effects on the fluxes of fluid and sodium, suggests that the transport mechanisms of fluid and sodium may be less interdependent than indicated by the relationship between their respective net movements.

The effect of enterotoxin on sodium fluxes seemed to indicate an inhibition of an uptake mechanism, since the effect was mainly on insorption. This might have been caused by inhibition of an exchange system such as that proposed by Turnberg, Bieberdorf and Fordtran (1969) where Na^+ ions are exchanged for H^+ ions. The result of this would be to decrease the sodium insorption and have no effect on sodium exsorption. At the same time, however, both insorption and exsorption of fluid would be decreased and this

was not observed in the present work.

However, if at the same time as inhibiting a $Na^+ - H^+$ exchange mechanism, the enterotoxin caused stimulation of another exchange system such as one in which Cl^- is exchanged for HCO^-_3 (Turnberg et al., 1969) then the increase in fluid fluxes resulting might be enough to reverse the above reduction and to produce an increase in both insorption and exsorption of fluid.

In order to explain the greater effect on fluid exsorption than insorption it is necessary to postulate a further stimulation of a secretory process, e.g. active secretion of chloride (Tidball, 1961) or even of water itself, although the weight of evidence is against active transport of water in the intestine (Schultz and Curran, 1968).

In the absence of further evidence, the above suggestions can only represent the most tentative of hypotheses, in order to explain results which by their complexity suggest that the processes involved are equally complex.

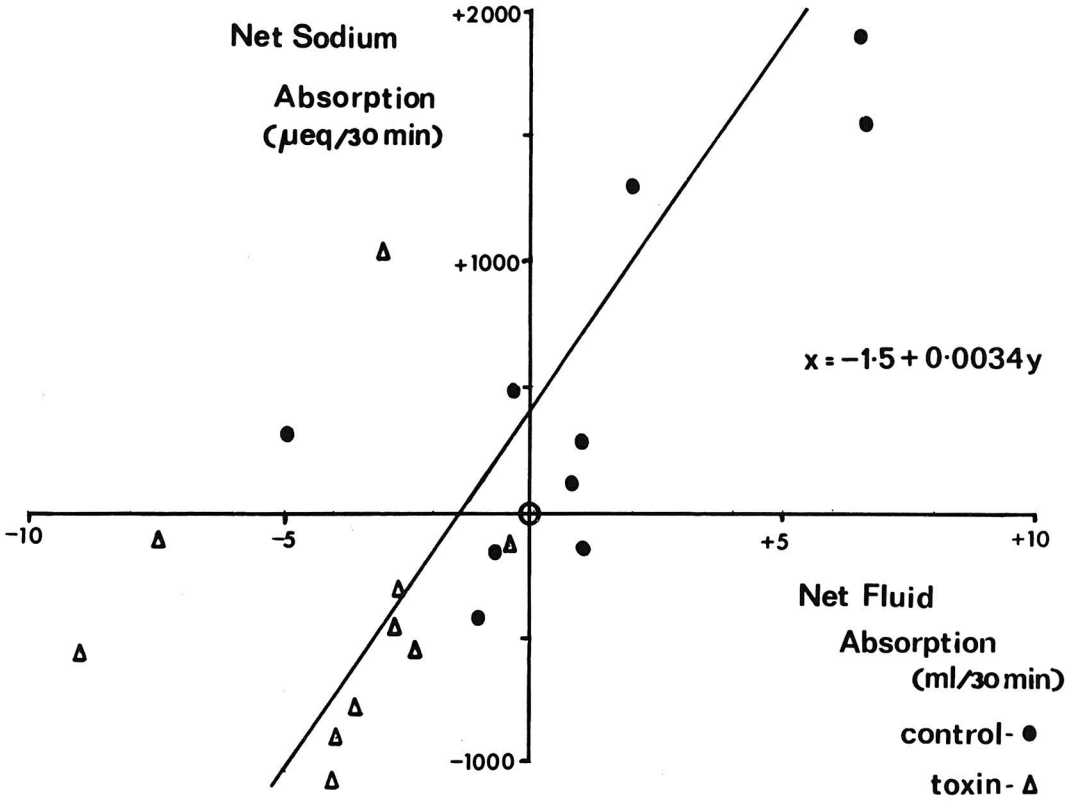
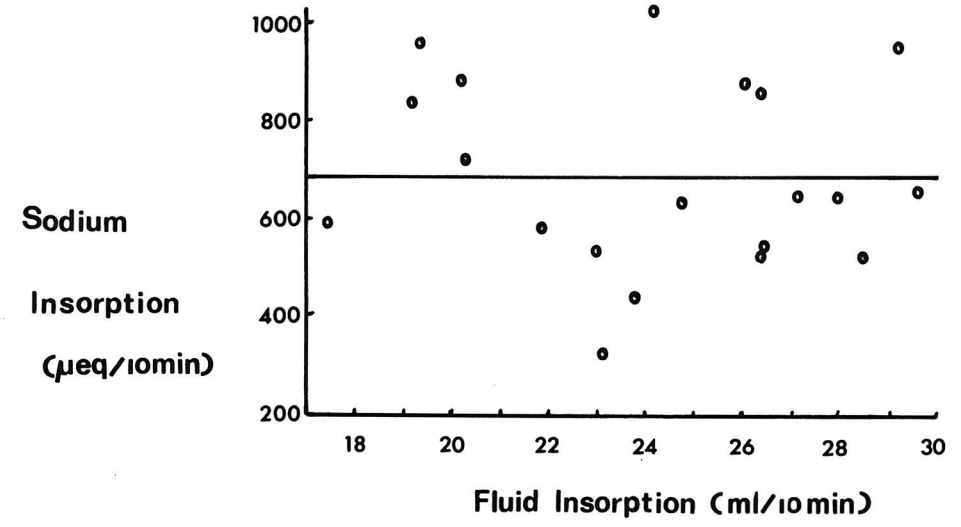


Fig.17 The relationship between net fluid and net sodium absorption from 10 Thiry-Vella loops. Each point represents the mean of at least 5 observations from either control (circles) or enterotoxic (triangles) solutions.

The calculated regression line is shown, and regression is significant ($P < 0.01$)



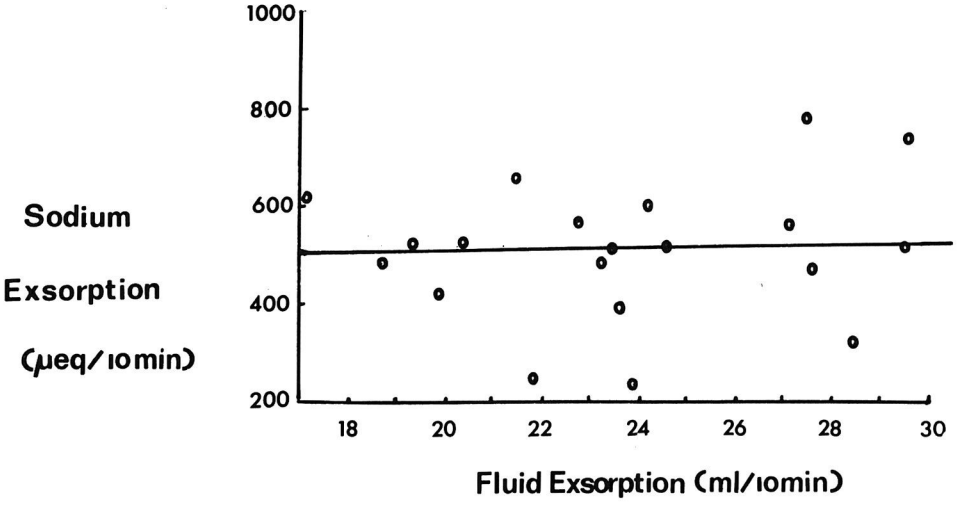


Fig.18 The relationship between unidirectional fluxes (upper-insorption; lower-exsorption) in 6 Thiry-Vella loops. Each point shows the mean of 5 observations under a particular set of experimental conditions.

The calculated regression lines are shown; in neither case is regression significant. ($P > 0.05$)

Implications of the possible isotope effect of deuterium oxide.

No adjustment has been made to the figures obtained for unidirectional fluxes to account for any deuterium oxide isotope effect (Appendix 3.)

The effect of enterotoxin on fluid insorption was small (although significant overall). Since the figures for exsorption were derived from these, then it is unlikely that any isotope effect present would qualitatively affect the results for enterotoxin activity.

An isotope effect would, however, quantitatively affect the figures obtained for unidirectional fluid fluxes. The result would be to underestimate the magnitude of the unidirectional fluxes. The amount of the underestimation would depend on the degree of the isotope effect. If deuterium oxide behaves in the same way as in the rat intestine *in vitro* then the unidirectional fluxes may be underestimated by up to 10%.

Even without any allowance of this nature, the size of the unidirectional fluxes is considerable in comparison with the net fluid movement. The loops in which these experiments were carried out were all from the distal ileum. It has been shown, in man, that the flux rates for fluid in the jejunum are not very different from those in the ileum (Whalen, Harris and Soergel, 1965), and if the calf small intestine behaves along its length in a similar manner to the loops, then the volume of the unidirectional fluxes to and from the small intestinal lumen may be estimated as 5.5 to 6.0 l/hr.

It has been found (Fayet, 1968a) that scouring calves lose about 0.9 l/day more fluid than do healthy calves. On the basis of the above estimate, the increased loss of fluid represents less than 1% of the volume of unidirectional flux during 24 hours. This indicates the small percentage change in balance of fluid fluxes which is necessary to produce considerable losses of fluid in the faeces.

3. THE EFFECT OF CULTURE FILTRATES FROM DIFFERENT STRAINS OF E.COLI ON FLUID MOVEMENT IN THIRY-VELLA LOOPS.

INTRODUCTION.

As well as the strains of E.coli mentioned in Section I, Dr. Williams Smith kindly provided cultures of a strain of E.coli which had been infected with the plasmid for enterotoxin production (F.11, P.215 Ent +ve) together with cultures of the same strain which had not been infected (F11,P.215 Ent -ve) (Smith and Halls, 1968). It seemed of interest to compare their activities on fluid movement in Thiry-Vella loops.

It has been suggested (Smith and Halls, 1967a) that only a small proportion of strains of E.coli isolated from field outbreaks of calf scour are enterotoxic (i.e. cause dilatation of ligated loops of intestine.) It seemed possible that the ligated loop test might be indicating only the most potent enterotoxin producers, and that a more sensitive test might indicate more widespread occurrence of enterotoxin production. Therefore, a series of strains of E.coli isolated from sick and healthy animals were tested for their ability to cause fluid production in Thiry-Vella loops.

MATERIALS AND METHODS.

The strains F11, P215 Ent +ve and F11, P215 Ent -ve were tested in the manner described earlier for the B 44 strain. Acetone extracts of culture filtrates were prepared, and these were placed in two Thiry-Vella loops for 30 min. periods, using the Ent -ve material as the control.

A further series of strains of E.coli were kindly supplied by Dr.W.J.Penhale. These were taken from three groups of animals:-

1.) Calves which had died from scour without septicaemia (18 animals). The organisms were smooth mucoid strains and were the predominant strain recovered from the upper small intestine.

2.) Calves which had died from colisepticaemia. The organisms were isolated from blood and organs either pre or post-mortem (8 animals).

3.) Healthy animals. These were rough strains and were taken from faecal swabs (6 animals).

Culture filtrates were prepared from these strains in the manner described earlier, but a different technique was used to test for enterotoxin activity:-

Two 45 minute absorption periods were used. During the first 45 minutes, 30ml of uninoculated culture medium filtrate (UCM.) was placed in the loop. At the end of the period, the remaining fluid was drained and removed as completely as possible with a syringe. The volume recovered was noted. During the succeeding 45 minute period, 30ml of the culture filtrate from the organism under test was placed in the loops, and again the fluid recovered at the end of 45 mins. was noted.

The difference between the absorption from control fluid (UCM.) and the fluid derived from the E.coli culture was taken as the exudate volume resulting from enterotoxicity.

Four Thiry-Vella loops (2 animals) were used in these experiments, and material from each strain was tested at least twice in different loops.

Of the 18 strains from scouring calves, 12 were sent to Dr. W.J.Sojka, C.V.L. Weybridge, who kindly examined them serologically, (O.K.grouping).

RESULTS.

1. The effect of culture fluid extract of Ent-ve and +ve strains on fluid movement from loops A and B is shown in Table (13).

Loop	Organism	Fluid absorption ml/30 min.	
A	Period 1.(Ent -ve)	-3.4 ± 1.0	P < 0.001
	Period 2.(Ent +ve)	-12.6± 2.4	
B	Period 1.(Ent -ve)	-1.2 ± 1.4	N.S.
	Period 2.(Ent +ve)	-4.4 ± 2.2	

Table (13) Fluid absorption during consecutive 30 minute periods from culture fluids from Ent. - ve and Ent. +ve strains of E. coli respectively.

Figures represent means of 5 experiments ± S.E. Negative signs show net secretion. Means were compared by paired t-test.

2. The fluid exudate in response to culture fluids from strains from healthy, scouring and septicaemic calves is shown in Table (14). The strain of E.coli, B44, for comparison, gave a mean volume of exudate of 16ml ± 1.2 in 20 experiments involving all four loops.

Control experiments showed that when uninoculated culture medium was placed in the loops, the volumes recovered after the two 45 min. periods differed by less than 3 ml.

Group of Calves from which organisms were taken.	Number of animals	Fluid Exudate ml/45 mins. ± S.E.	
Healthy	6	11.3±1.6	$p < 0.01$
Scouring	18	19.3±1.1	
Septicaemic	8	10.6±0.8	$p < 0.001$

Table (14) Fluid exudate response to culture filtrates from different groups of calves. The fluid exudate response is the difference between the absorption from control and culture fluids during consecutive 45 min. study periods. Differences between groups were tested by 'Students' t-test.

Serological examination of 12 of the strains from scouring calves showed that 8 of these possessed antigenic similarity with strains described by Smith and Halls (1967a) as enteropathogenic for calves. One further strain was antigenically similar to one normally associated with the avian species. The final 3 were untypable with the available diagnostic sera.

DISCUSSION.

The Ent +ve and Ent -ve strains gave inconsistent results in the loops in which they were tested - the difference being significant in one but not in the other. It appeared that the Ent -ve culture filtrate possessed some ability to provoke secretion.

The results from the experiment where organisms isolated from scouring calves were compared with those from septicaemic and healthy calves showed that the organisms from the scouring calves provoked significantly greater amounts of secretion than did those from either of the other two groups.

This could indicate that the strains from scouring calves possessed a greater degree of enteropathogenicity, and it is noteworthy that material from both of the other groups possessed some ability to provoke secretion.

This, together with the results from the Ent -ve organism may indicate that enterotoxin production is not an all-or-nothing phenomenon, and that non-pathogenic strains may produce small amounts of enterotoxic substances. A similar suggestion has been made with respect to Vibrio cholerae, (Blachman, Basu and Pickett, 1970) where evidence has been presented to show that 'non-pathogenic' strains may produce small amounts of enterotoxin.

The serological evidence suggests that at least eight of the strains from the scouring calves possessed antigenic similarity with strains associated with enteropathogenicity for calves. The significance of this is difficult to assess, since the serology of E.coli strains pathogenic for calves is not well established. Moreover, the infective nature of the capacity to produce enterotoxin (Smith and Halls, 1968) may further reduce the significance of serological findings.

Nevertheless, the presence of the eight strains with antigenic similarity to strains previously associated with the condition is striking. It may be that these strains not only possess the ability to produce enterotoxin, but also have the ability to become established and multiply in the upper small intestine (Smith and Halls, 1967a).

Most of the group of scouring calves had been obtained from dealers' premises, and so the high incidence of enterotoxin producing strains suggests that such strains may be more common than suggested by Smith and Halls (1967a).

SECTION III

SOME PROPERTIES OF ENTEROTOXIN ACTIVITY IN THIRY-VELLA LOOPS IN CALVES.

INTRODUCTION.

Thiry-Vella loops provide a convenient preparation for studying some of the properties and characteristics, so far only poorly defined, of E.coli enterotoxin.

Since bacterial endotoxin (lipopolysaccharide) may be released into culture media under some circumstances (Guckian and Perry, 1966; Marsh and Crutchley, 1967) and moreover it has been suggested that soft-agar culture fluids contain appreciable amounts of endotoxin (Wray and Thomlinson, 1969a), it seemed important to show that enterotoxin activity was separate from endotoxin.

Thiry-Vella loops may also be used to examine other properties of enterotoxin including:- the recovery of absorptive ability after enterotoxin activity; the dose-response relationship between enterotoxin and fluid exudate; the heat stability, systemic absorption and drug susceptibility of enterotoxin.

Of these properties, the dialysability and heat stability of enterotoxin have been examined by others. However two reports on the dialysability of enterotoxin are contradictory, (Smith and Halls, 1967b; Kohler, 1968) and so further experiments are needed. The reports on heat stability (Smith and Halls, 1967b; Truszczynski and Pilaszek, 1969; Kohler, 1968) also show some inconsistencies.

The experiments to be described were carried out in order to provide information on the properties mentioned above.

1. Distinction between Enterotoxin and Endotoxin (Bacterial Lipopolysaccharide).

Methods.

In order to assess the amount of endotoxin in acetone extracts of culture fluids, the toxicity of a concentrated solution of extract was compared in mice with a preparation of endotoxin prepared by phenol-water extraction.

The concentrated solution of acetone extract of culture fluid was obtained by dissolving the acetone extract from 300ml of culture fluid in 20ml of sterile distilled water. The solution was injected intraperitoneally into groups of mice (5 animals per group) and the $L.D._{50}$ was calculated using the method of Reed and Muench (1938).

Phenol-water extracted endotoxin was obtained in a freeze-dried state from the *E.coli* strain B44 by the method of Westphal, Luderitz, Eichenberger and Keiderling (1952). The $L.D._{50}$ of this preparation for mice was determined as above.

The effect of endotoxin on net fluid, electrolyte and glucose absorption from Thiry-Vella loops was determined by dissolving the freeze-dried material in test solution II (page 52) and comparing the absorption from this solution with absorption from solution II alone during consecutive 30 minute periods.

Results.

The $L.D._{50}$ for mice of a concentrated solution of enterotoxic acetone extract was found to be equivalent to 1.28ml of the concentrated solution. This is equivalent to 19.4 ml of the standard solution of enterotoxin (i.e. acetone extract derived from 30ml of culture filtrate and redissolved in 30ml of test solution I).

The $L.D._{50}$ for mice of the phenol-water extracted endotoxin was 1.3mg.

Therefore, even if all the toxicity of the acetone extract of culture fluid resulted from its endotoxin content, then 30ml of the standard enterotoxic solution was unlikely to contain more than 2mg of endotoxin.

The effect of 5mg of endotoxin on net absorption in two loops is shown in Table (15). In no case was a significant effect on absorption seen. A dose of 25mg was also found to be without effect when tested once in each of two loops.

Loop		FLUID ABSORPTION ml per 30 min.	SODIUM ABSORPTION μ eq per 30 min.	POTASSIUM ABSORPTION μ eq per 30 min.	GLUCOSE ABSORPTION Mg/30 min.	BICARBONATE ABSORPTION μ eq per 30 min.
E	ENDOTOXIN	-3.8 ± 1.0	-550 ± 150	-53 ± 6	+85 ± 3	-66 ± 9
	CONTROL	-4.6 ± 0.8	-580 ± 110	-53 ± 6	+72 ± 4	-14 ± 89
F	ENDOTOXIN	-2.9 ± 0.2	-350 ± 60	-33 ± 20	+58 ± 4	+25 ± 40
	CONTROL	-2.7 ± 0.6	-360 ± 70	-34 ± 90	+45 ± 12	-20 ± 80

Table (15) The effect of phenol-water extracted endotoxin (5mg) on net absorption of fluid, sodium, potassium, glucose and bicarbonate in two loops. Figures represent the means of 5 observations ± S.E. Positive signs indicate net absorption, negative signs indicate net secretion.

2. Recovery of Thiry-Vella loops after exposure to enterotoxin.

Methods.

Absorption of fluid from test solution II was measured during 30 minute periods before and at intervals after exposure to the standard amount of enterotoxin (i.e. that derived from 30ml of culture filtrate) for a 30 minute period. The experiments were carried out in three loops, all of which were from the distal ileum.

Results.

In all three loops, (Fig 19) the ability to absorb fluid recovered within a period of 5 hours. In loop B, recovery appeared to be very rapid.

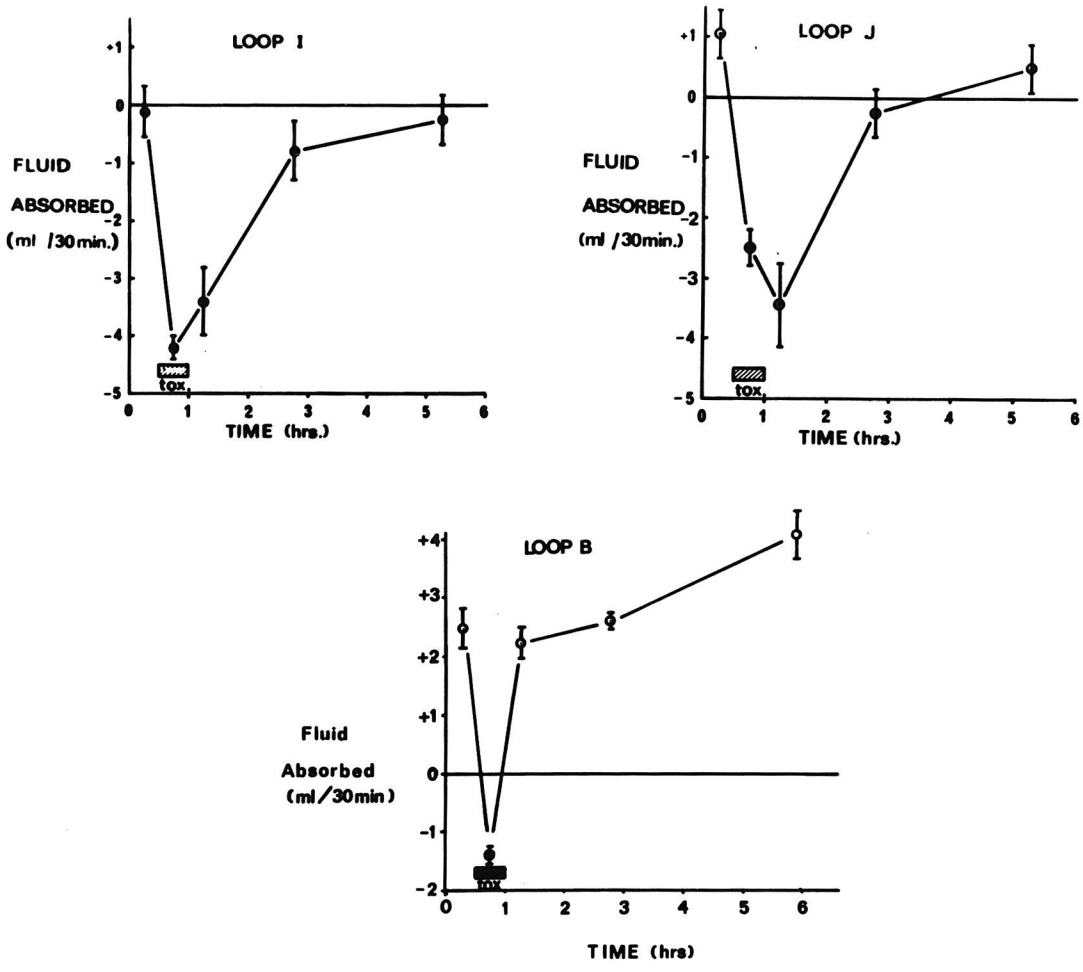


Fig.19 Recovery of fluid absorption after exposure to enterotoxin.

Absorption of fluid from test solution in loops I,J & B is shown before and after exposure to one standard dose of enterotoxin for 30 minutes. Each point represents the mean of at least five observations.

3. Dialysis and Ultrafiltration of Enterotoxin.

Methods.

a) Effect of dialysis for 48 hrs.

240ml of enterotoxic culture filtrate was prepared by culturing the E.coli strain B44 on soft agar and separating the culture fluid as described earlier.

Acetone (2040ml) as added and the precipitate was allowed to settle overnight at -30°C. After decanting the supernatant acetone, the precipitate was dried and redissolved in 20ml of sterile distilled water. The redissolved extract was spun at 4000 r.p.m. for 10 minutes to remove any undissolved material.

5ml amounts of the concentrated extract were placed in Visking dialysis tubing and were dialysed for 48 hours at 4°C against 200ml distilled water. At the end of this time, the dialysates and dialysands were freeze-dried.

Uninoculated culture medium was treated in an identical manner as a control.

In order to test for enterotoxic activity, 250mg amounts of dialysate or dialysand were added to 30ml of test solution II and their effect compared with 250mg of the corresponding control material. Osmolalities of the test and control solutions were determined cryoscopically.

b) Effect of dialysis for 7 days.

To examine the proportion of activity that was dialysable over a longer period, 5ml amounts of concentrated enterotoxic extract were prepared as above. In this case, the sacs were dialysed against 200ml volumes of distilled water at 4°C but with daily changes of water for seven days.

c) Ultrafiltration experiments.

210ml of culture filtrate from the B44 strain of E.coli were precipitated by 8 volumes of acetone and the precipitate treated as shown in Fig.(20). Uninoculated culture medium was treated in a similar manner to provide control material.

The membranes used were Amicon PM-10 (Amicon Ltd, High Wycombe,Bucks.) with a pore size such that the cut-off was at approximately 10,000 M.W., and Amicon UM-2 where the cut-off was at approximately 1000 M.W.

Ultrafiltration was carried out under nitrogen (20-40 p.s.i.) in a cell fitted with a magnetic stirrer to prevent clogging of the membrane.

The residue from the PM-10 membrane (fraction D), the residue from the UM-2 membrane (fraction E) and the filtrate from the UM-2 membrane (fraction F) were all freeze-dried.

Samples from the three fractions were added to test solution II and absorption from enterotoxic fluid was compared with that from fluid containing equal weights of corresponding material from uninoculated culture medium during consecutive absorption periods in Thiry-Vella loops. The weights of material tested were 120mg (fraction D) 100mg (fraction E) and 50mg (fraction F).

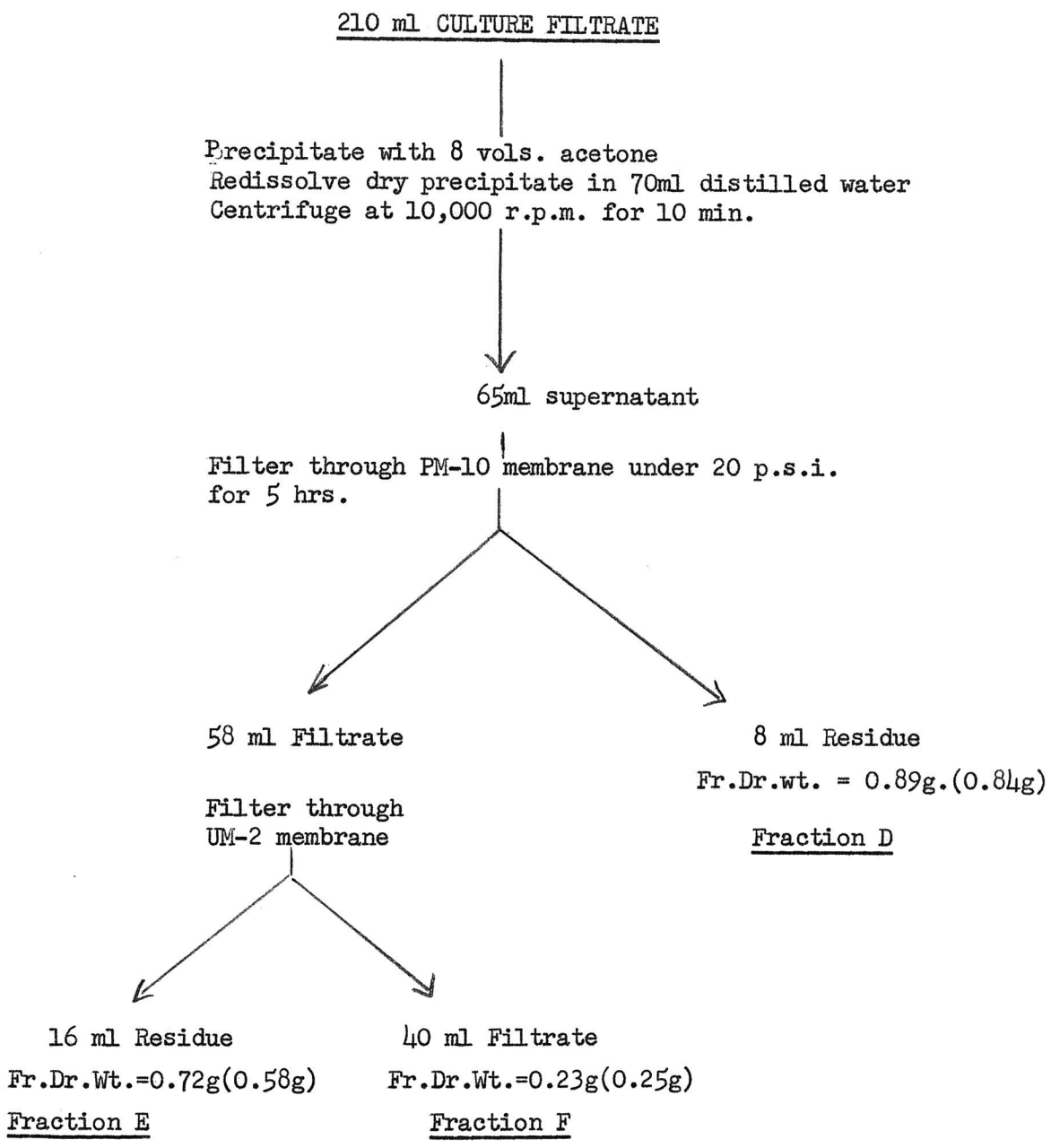


Fig. (20) Preparative procedure for ultrafiltrate fractions of culture fluid.

Weights of fractions in parenthesis refer to the weights of freeze-dried material obtained from equal volumes of uninoculated culture medium(control). Fr. Dr. = Freeze-Dried.

Results.

a) Dialysis for 48 hours.

The weight of freeze-dried material obtained from the different sources were as shown below:-

	Dialysand	Dialysate
Enterotoxic extract	1.47 g.	1.5 g.
Uninoculated culture medium	1.30 g.	1.3 g.

The effect of 250mg of freeze-dried dialysate on fluid, sodium, bicarbonate and chloride movement is shown in Fig. (21). The effect of 250mg of dialysand is shown in Fig.(22). In both cases the effect on all four parameters was significant. Differences in osmolality between test and control solutions were negligible.

b) Dialysis for seven days.

Fig. (23) shows the effect on fluid absorption of 250mg of dialysand recovered after 7 days dialysis against 7 changes of distilled water. The control material was extracted from uninoculated culture medium treated in the same way. Most of the enterotoxic activity was lost and the difference between the fluid absorption during control and test periods was not significant. In this case, electrolyte movement was not tested.

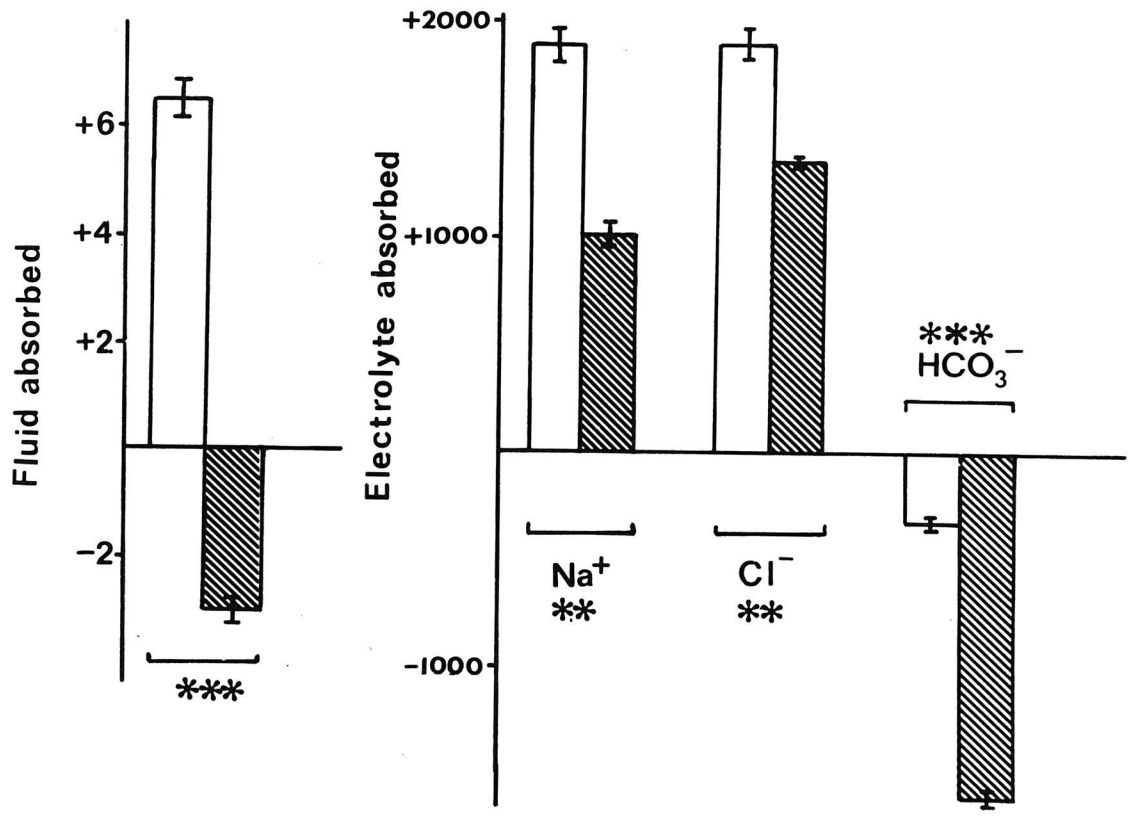


Fig.21 The effect of 250 mg of freeze-dried dialysate (48 hours dialysis, hatched columns) on net absorption of fluid (ml/30 min.) and electrolyte (μeq./30min.) from test solution II during consecutive 30 min. periods in loop P. 250 mg of freeze-dried dialysate of uninoculated culture medium was used as a control (open columns).

Each column represents the mean of 5 observations. Vertical bars show standard errors. Positive signs indicate net absorption, negative signs indicate net secretion.

Differences between the two periods were tested by paired t-test. (*P < 0.05; **P < 0.01; ***P < 0.001).

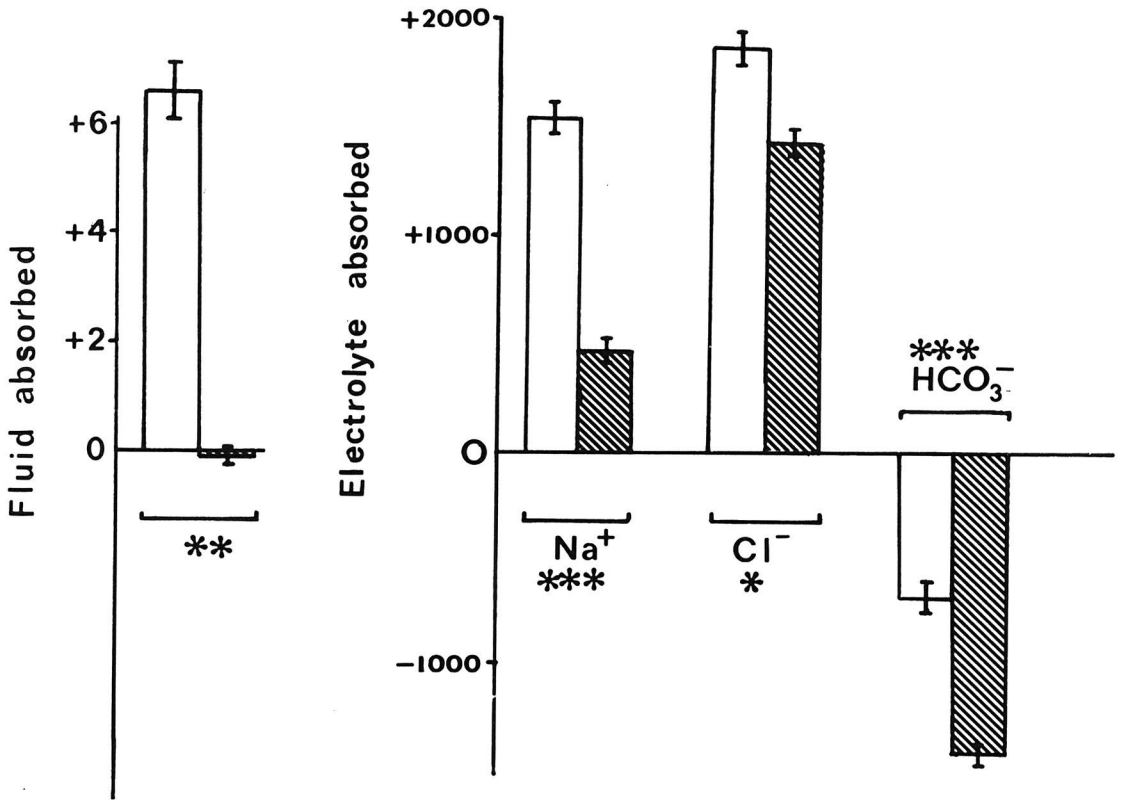


Fig.22 The effect of 250 mg of freeze-dried dialysand (48 hours dialysis, hatched columns) on net absorption of fluid (ml/30 min.) and electrolyte (μeq/30 min.) from test solution II during consecutive 30 min. periods in loop 0. Open columns show results from control material.
Notation is as in Fig 18.

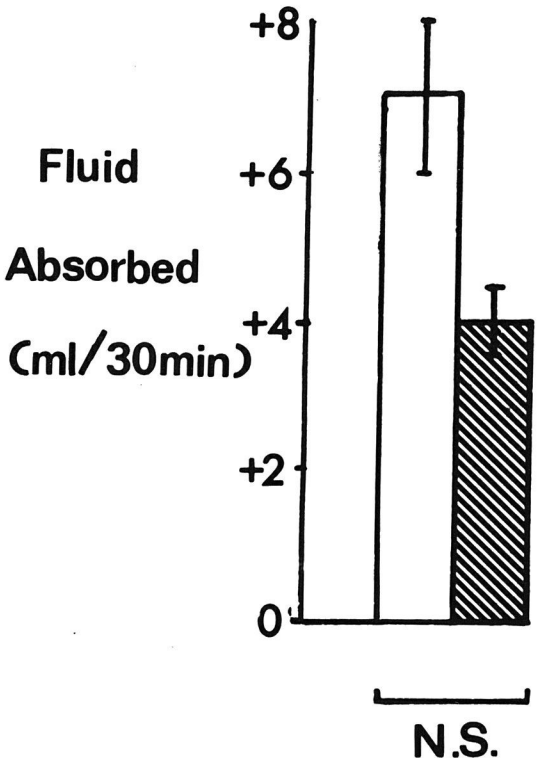


Fig. 23 The effect of 250 mg of freeze-dried dialysand (7 days dialysis, hatched column) on net fluid absorption from test solution II in loop 0. The open column shows absorption from control material. The difference is not significant. (N.S. $P > 0.05$)

c) Ultrafiltration experiments.

The hatched histograms in Fig. (24) show the difference between the mean fluid absorption during control periods and the subsequent test periods when the loops contained fractions from enterotoxic filtrate. The differences seen were attributed to enterotoxin activity and were referred to as exudate. The loops used were O and P (distal ileum).

The clear histograms, T1, T2 and T3 represent the theoretical response of the loops to the amount of enterotoxin in the three cases:

T1 - if all the toxic activity is retained by the PM-10 membrane.

T2 - if all the toxic activity is retained by the UM-2 membrane, but none by the PM-10.

T3 - if neither the PM-10 or UM-2 membranes retain enterotoxic activity.

These figures were calculated from the means of at least three previous responses of the loops to standard doses of enterotoxin (i.e. that obtained from 30ml of culture filtrate) given that the total amount of enterotoxic culture filtrate at the start of the experiment was 210ml (7 standard doses).

Material from each fraction was tested at least twice, and the osmolalities of the solutions were checked in each case. Differences in osmolalities between solutions of control and test material were small, but where they were found, adjustments were made to correct them.

The results showed that the responses obtained corresponded most closely to the theoretical case T2, indicating that the molecular weight of most of the active material lay in the 1000-10,000 range. However some activity was found in fraction F (less than 1000 M.W.) and it was found that this was significant ($p < 0.05$) although the amount involved was small.

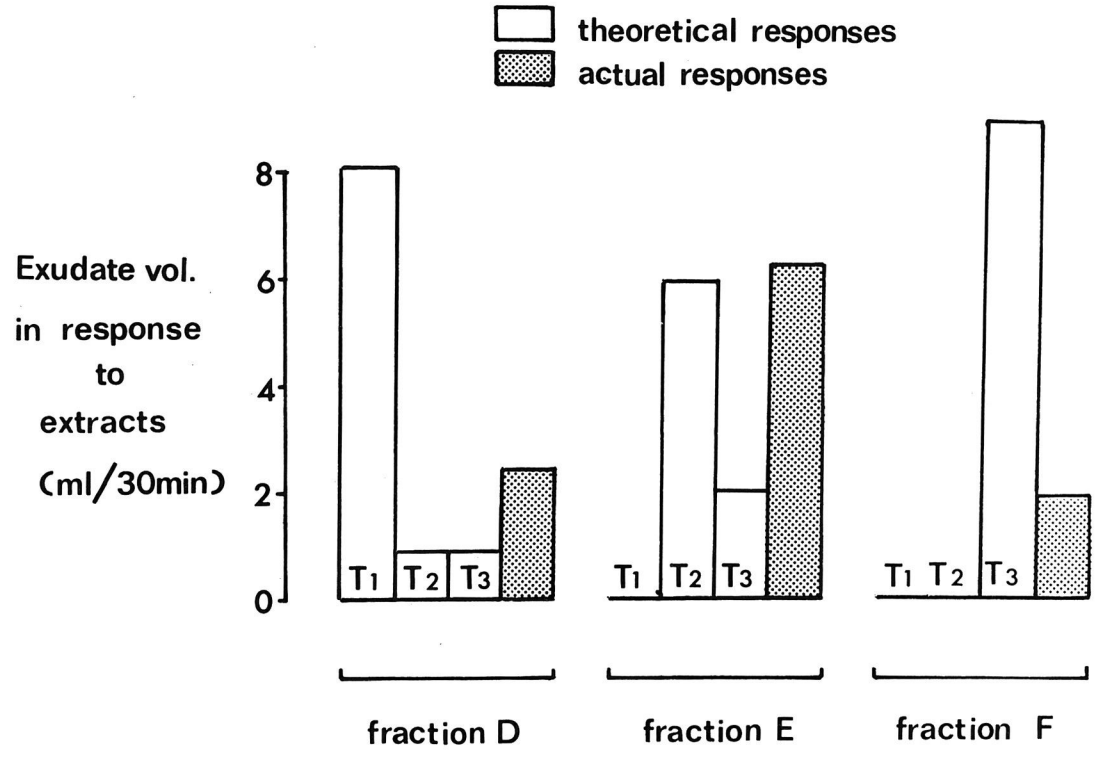


Fig. 24 The fluid exudate response to ultrafiltrates of enterotoxic culture filtrate. (Exudate=difference between absorption from control and enterotoxic solutions.)

Fraction D- residue > 10,000 molecular weight
Fraction E- residue > 1,000 " " but < 10,000
Fraction F- filtrate < 1,000 " "

The stipled columns show the responses obtained, each being the mean of at least two observations.

The clear columns represent the theoretical response (calculated on the past performance of the loops) which would be expected if:-

T1- Molecular weight of enterotoxin was > 10,000.
T2- " " " " " > 1,000 but < 10,000
T3- " " " " " < 1,000.

4. Dose-Response relationship of enterotoxic material.

Methods.

1. Using enterotoxic acetone extract.

In an initial series of experiments, crude acetone extract of enterotoxic culture filtrate was added to solutions of sodium chloride containing polyethylene glycol 4000 as a marker. Differing amounts of acetone extract were added to solutions containing calculated amounts of NaCl to make the solution isotonic.

30ml amounts of the solutions were then placed in a Thiry-Vella loop during 30 minute periods separated by 45 minute recovery times. The exudatory response (i.e. the difference between absorption from control and enterotoxic solutions) was measured in each case.

2. Using freeze-dried enterotoxin.

In these experiments, 25-200mg amounts of freeze-dried dialysate of enterotoxic material were dissolved in 30ml amounts of test solution I (page 52). The osmolality of the resulting solution was then checked and adjusted to isotonicity by addition of appropriate amounts of sodium chloride. The solutions were tested as above, but at least 24 hours was allowed for recovery after each experiment.

Results.

1. Using enterotoxic acetone extract.

It was found that no relationship could be shown between dose and response using this material, so this approach was abandoned.

2. Using freeze-dried dialysate.

The results from a series of experiments is shown in Fig (25). It can be seen that log. doses from 25 to 100mg of enterotoxic material appear to show a linear relationship with the exudatory response, but that the response to 200mg is not linearly related to the other points.

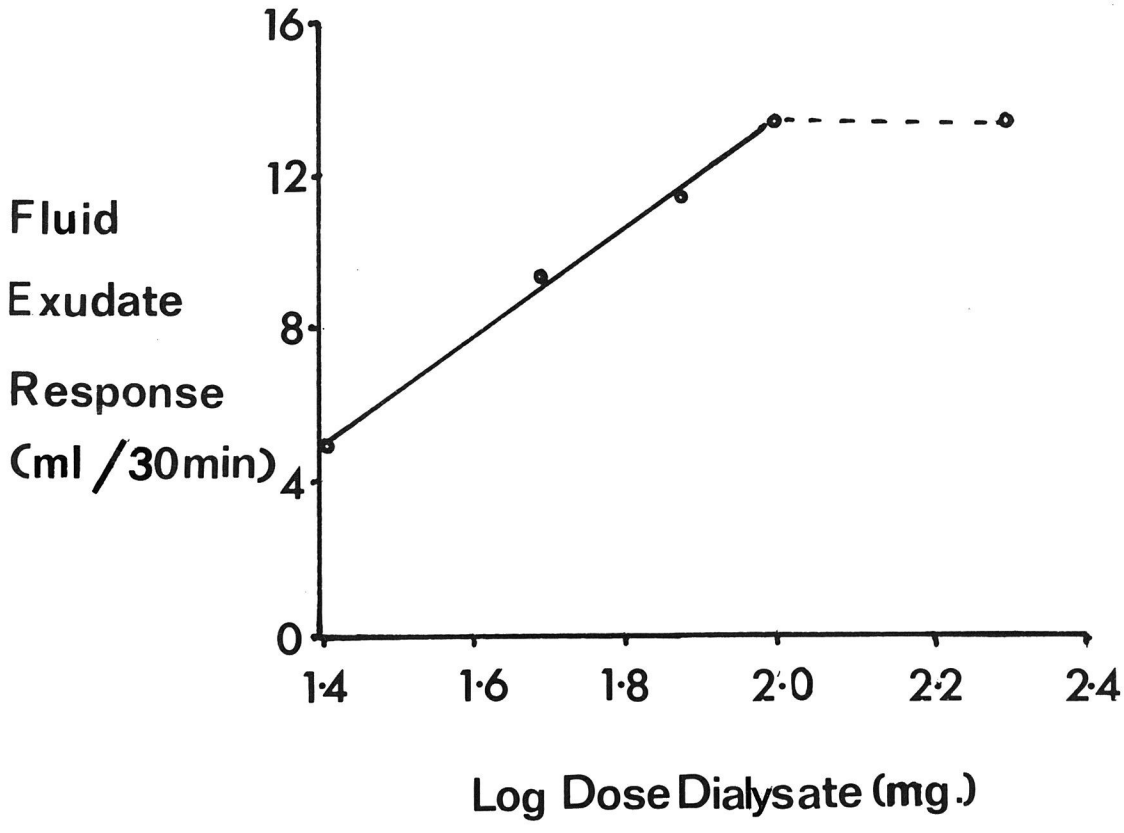


Fig. 25 Log dose-response relationship between the dose of freeze dried dialysate and fluid exudate response. The exudate is the difference between absorption from control and enterotoxic solutions.

5. Heat Stability of Enterotoxin.

Methods.

30ml amounts of enterotoxic culture filtrate were heated at temperatures of 90°C and 100°C for 45 minutes, and at 121°C for periods of 45 minutes and 2 hours. Acetone extracts were prepared from the culture filtrates and from uninoculated culture medium. The extracts were dissolved in test solution I and absorption from the two solutions was tested in Thiry-Vella loops during consecutive 30 minute periods.

The difference between the fluid absorption from control and test solutions was taken as being due to enterotoxin activity, and was referred to as the exudate.

Results.

Heating at 90°C or 100°C for 45 minutes was found to have no effect on the volume of fluid exudate when tested in Thiry-Vella loops. Heating at 121°C for 45 minutes was without effect on enterotoxin activity, but heating at 121°C for 2 hours caused a reduction in the fluid exudate (Table 16).

	Exudate (ml. /30 min.)
Control response (extract heated to 65°C)	6.4
Extract heated at 121°C for 45 min.	6.3
Extract heated at 121°C for 2 hrs.	2.8

Table (16) Heat stability of enterotoxic extracts. Figures are the means of experiments on loops P, U and V. Material treated in each manner was tested at least twice in each loop.

6. Systemic Absorption of Enterotoxin.

Methods.

Two calves were used, each of which had two Thiry-Vella loops made from adjacent loops of intestine (loops O and P in one animal, U and V in another). In each calf, absorption of fluid and sodium was measured from control and enterotoxic material placed in one of the two loops during successive 30 minute study periods. Simultaneously, absorption from test solution II alone placed in the second loop was measured during the same 30 minute periods. In the absence of enterotoxin absorption, this absorption from test solution II should be unchanged during the second 30 minute period in comparison with the first.

In the first calf, (loops O and P) one standard dose of extract (that produced from 30ml of culture filtrate) was placed in loop O and the effect on absorption from loop P was examined.

In the second calf (loops U and V) two standard doses of extract were placed in loop U, and the effect on absorption from loop V was examined.

Results.

The effect of the presence of one standard dose of enterotoxin in loop O on net absorption of fluid and sodium from loop P are shown in Fig.(26). The effect of two standard doses of enterotoxin in loop U on net absorption of fluid and sodium from loop V is shown in Table (17).

The net absorption of fluid and sodium from loop P is significantly affected by the presence of enterotoxin in loop O. This could not be confirmed in loops U and V, where the change in fluid and sodium movement in loop V resulting from the presence of enterotoxin in loop U was not significant.

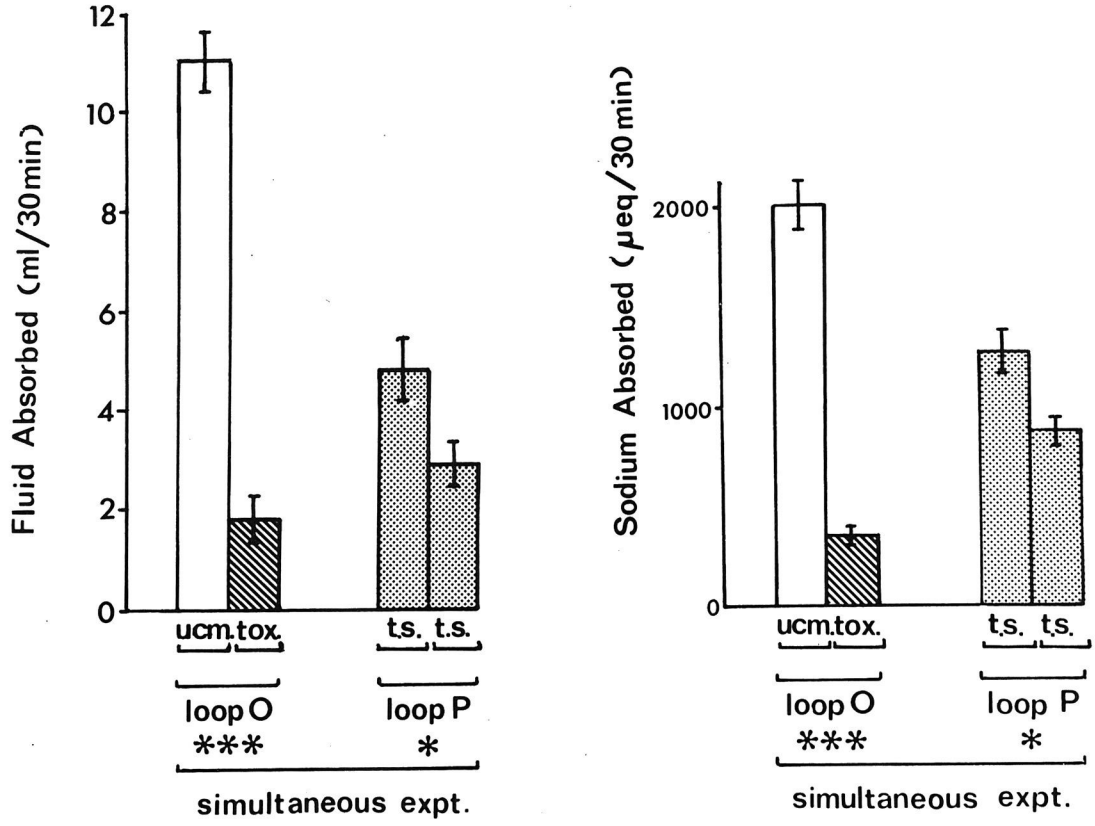


Fig. 26 The effect of enterotoxin (1 standard dose, hatched columns) in loop O on net fluid and sodium absorption from test solution II in loop P (stipled columns.) Open columns show absorption from control material.

Each column respresnts the mean of 5 observations. Vertical bars show standard errors. Differences were tested by paired t-test. (* $P < 0.05$; *** $P < 0.001$)

		FLUID (ml/30min)		SODIUM (μeq/30min)	
Loop U	Control (Uninoculated culture medium)	+1.72±0.75	$p < 0.05$	+760±140	$p < 0.05$
	Toxin (2 standard doses)	-0.76±0.32		+20 ±160	
Loop V	Test sol II	+2.78±1.4	N.S.	+600±210	N.S.
	Test sol II	+1.98±0.8		+320±130	

Table (17) The effect of 2 standard doses of enterotoxic material in Loop U on net fluid and sodium absorption from Loop U and Loop V. Figures represent means ± S.E. of five experiments. Differences were tested by paired t-test.

7. Susceptibility of enterotoxin activity to inhibition by drugs.

Methods

The drugs used were either added to the test solution I before addition of control and enterotoxic material (1 standard dose), or were given parenterally before the experiment.

The drugs used, their source, dose and route of administration are shown below:

Drug	Source	Route of Admin.	Dose
Mepyramine maleate	British Drug Houses	Intraluminal	10µg/ml test sol.
		Intramuscular	7.5 mg/kg.
Methysergide bimaleate	Koch-Light	Intraluminal	100µg/ml test sol.
Acetazolamide sodium	Lederle	"	0.5 amd 2.0 mg/ml test sol.
Betamethasone phosphate	Glaxo	Intravenous	0.2 mg/kg.

Results

Each drug was tested at least twice at the above doses, and in no case was inhibition of the enterotoxic effect on fluid movement observed.

DISCUSSION

Endotoxin has been shown by other workers to cause no dilatation in ligated loops of pig intestine (Smith and Halls, 1967b; Truszczynski and Pilaszek 1969). The present experiments confirm that endotoxin has no effect on calf intestine when tested in amounts greater than those found in culture fluids. This is despite the exceptional sensitivity of calves to the systemic effects of endotoxin (Penhale, 1965).

The effect of a single exposure of the Thiry-Vella loops to enterotoxin produces only a transient effect on fluid absorption. This implies that the changes caused by the presence of enterotoxin for a 30 minute period are relatively mild and easily reversed on removal of the toxin. The recovery period is considerably less than that found by Swallow, Code and Freter (1968) who used cholera enterotoxin in Thiry-Vella loops in dogs. They found recovery to be complete in a period approaching 96 hours, but used a one hour incubation period. The difference may also be due to greater potency of V.cholerae enterotoxin in comparison with E.coli enterotoxin.

The dialysis experiments showed that insignificant amounts of enterotoxic activity remained in the dialysand after 7 days dialysis, and that activity can be found in the dialysate. This is largely in agreement with the work of Kohler (1968) who showed that on prolonged dialysis, culture filtrates lost most, but not all, of their ability to cause diarrhoea in piglets. However, Smith and Halls (1967b) found no loss of dilating ability of culture fluids after dialysis, but did not test for activity in dialysate, and so their experiments were in this respect incomplete.

The fact that the enterotoxic material was dialysable suggested that the

molecular weight was probably less than 10,000 but a closer approximation was obtained by ultrafiltration.

These latter experiments showed that most of the enterotoxic activity lay in the 1000-10,000 M.W.range, since in Fig.(24) the responses obtained correspond most closely to those of the theoretical case T2 rather than either T1 or T3. However, fractions D and F both appeared to contain more activity than they would have if case T2 was the full explanation. The greater activity in fraction D than forecast by T2 for this fraction may be a result of the fact that the enterotoxin batch from which the fractions were obtained differed from that on which the calculation for T1, T2 and T3 were based. In general, batches of culture filtrate appeared to differ little in their enterotoxin content, but in this case it may be that fractionated batch (210ml) contained more than 7 x the standard dose.

The significant activity in fraction F cannot be explained simply in terms of theoretical case T2, since, whatever the potency of the preparation, no activity should pass the UM-2 membrane if T2 is correct.

Therefore it appears that little if any enterotoxic activity is retained by the PM-10 membrane, while most, but not all, of the activity is retained by the UM-2 membrane. The molecular weight of the enterotoxic fraction thus appears to lie in the 1000-10,000 range, but the presence of some activity in the final filtrate may either indicate some material with a molecular weight less than 1000, or alternatively may simply reflect imperfections in the precision of the cut-off point of the UM-2 membrane.

The properties described suggest that ultrafiltration may be a useful method of removing both large and small molecular weight contaminants in an initial step in purification of heat-stable enterotoxin.

The results of the experiments to test heat stability confirm that the material is very resistant to the effects of heat. No loss of activity was found on heating at 100°C, so confirming the observation of Smith and Halls (1967b), but contrasting with the observations of Kohler (1968) and Truszczynski and Pilaszek (1969) who found a partial loss of activity at this temperature.

Heating at 121°C for 45 minutes produced no loss of activity, despite the reports of Smith and Halls (1967b), Kohler (1968) and Truszczynski and Pilaszek (1969) that the ability to cause dilatation of ligated loops and diarrhoea in piglets was lost at this temperature. However, heating at 121°C for 2 hours caused a partial loss of activity. The explanation for these results may lie in the methods used to detect enterotoxin activity, since Thiry-Vella loops may be more sensitive to its effects than either of the methods previously used. Moreover the dose of enterotoxic material used (one standard dose) was probably greater than that necessary to produce a maximal exudate response (see below). In this case, partial loss of activity would not be reflected in a corresponding loss of exudate response.

A dose-response relationship was found to exist between the amount of freeze-dried dialysate and the fluid exudate response. No such relationship could be shown using crude acetone extract. This may have resulted from the relative crudity of the preparation which meant that considerable adjustment was necessary to allow for osmotic effects. The experimental design may also have allowed insufficient time for recovery of loops after enterotoxic activity.

The experiments using enterotoxic dialysate material allowed adequate recovery time, and the material was devoid of large molecular contaminants.

The fact that the response reached a maximum at the 100mg dose may reflect the relatively short period during which secretion was measured. If a longer period was allowed, then the 200mg dose might have produced a greater effect than the 100mg dose. 100mg was approximately equivalent to 18ml of culture filtrate, and suggests that the 'standard-dose' of enterotoxin (that derived from 30ml of culture filtrate) was greater than necessary to produce a maximal response.

The presence of a standard-dose of enterotoxin in loop O was associated with significant changes in net absorption of fluid and sodium from loop P. This implied that enterotoxin had been absorbed and was reaching the second loop via the circulation. A similar absorption of cholera enterotoxin has been demonstrated in other species (Serebro, Hendrix, Iber, Royall and McGonagle, 1968; Vaughan Williams and Dohadwalla, 1969).

However, the absorption of coli enterotoxin could not be demonstrated in loops U and V, where twice the dose of toxin in loop U had no significant effect on absorption from loop V. This may be explained on the basis of the exceptional sensitivity of loops O and P to enterotoxin activity, while loops U and V were very much less sensitive. It is therefore possible that the amount of enterotoxin absorbed from loop U was insufficient to cause an effect in loop V.

If some absorption of enterotoxin was occurring as suggested from the behaviour of loops O and P, then this would imply that absorption in the upper intestine may be affected by the presence of enterotoxin producing organisms in the lower intestine, without the presence of micro-organisms in the former region. However, this can not be said to be proven, since the second group of experiments did not confirm the finding.

In the doses used, none of the drugs tested possessed inhibitory activity against enterotoxin. This gives no support to the use of related compounds in diarrhoeal conditions in which enterotoxin may be involved. However, in higher doses than those tested, effects may become apparent.

Acetazolamide intraluminally was apparently without effect, although the inhibition of cholera enterotoxin in rabbit loops (Norris et al., 1969) was obtained using animals pretreated with 20-50mg/kg of acetazolamide sodium as well as 0.5 mg/ml added to the test solution. Comparable amounts administered to calves would have been prohibitively expensive. The possibility must remain that with such higher doses an effect may have appeared.

SECTION IV

LACTASE ACTIVITY IN INTESTINAL MUCOSA AND ITS RELATIONSHIP WITH DIARRHOEA AND COLI ENTEROTOXIN.

INTRODUCTION.

In their study of the composition of faeces from scouring calves, Blaxter and Wood (1953) found large amounts of volatile fatty acids together with a low pH. They suggested that this may be due to a primary disfunction high in the alimentary tract which allowed undigested carbohydrate to reach the large intestine where it was then fermented by microorganisms. Smith (1962) noted the undigested appearance of food material in the small intestine of scouring calves, and suggested that an enzyme deficiency might be responsible. Although he did not refer to a particular enzyme, lactase (β galactosidase) deficiency could account for the changes seen.

Kwiatkowski (1967) described lactase deficiency in calves with 'digestive-tract necrosis' and diarrhoea on Polish farms. It is not clear how closely this disease corresponds with neonatal diarrhoea in calves as encountered in the United Kingdom.

In view of the above observations, together with those referred to earlier, lactase activities of intestinal mucosa of calves were examined. Material from scouring animals was compared with that from a group of normal animals.

Despite the evidence that lactase activity in ligated loops was unaffected by twelve hours contact with enterotoxin, (Section I) an attempt was made to determine whether further prolonged contact between enterotoxin and gut mucosa

might result in depression of lactase activity. The effect of enterotoxic culture filtrates in vitro on lactase activity was also examined.

Oral feeding of high levels of lactose has been found to be associated with diarrhoea in calves (Rojas, Schweigert and Rupel, 1948; Huber, Jacobson, McGilliard and Allen, 1961.) An effort was made in the present experiments to determine the level of dietary lactose which would cause changes in faeces consistency in two healthy calves. It was hoped that this might give information on the reserve capacity of lactase activity in normal animals.

Lactose tolerance tests were also carried out in normal and diarrhoeic calves in order to gain indirect information on the intestinal lactase activity of these animals.

MATERIALS AND METHODS.

(1) Lactase activity in mucosa from healthy calves and calves dying after scour.

Samples of intestine from ten healthy calves were obtained from the abattoir. The calves were Friesian or Ayrshire bull calves and were carefully examined to exclude any diarrhoeic animals. The average age was 6 days. The intestine was removed immediately after the animals had been killed and bled, and the mesentery was cut along its length to allow segments to be removed from 5%, 25%, 50%, 75% and 98% of the distance between pylorus and ileo-caecal valve. The material was carried to the laboratory in iced saline.

Samples of intestine from calves which had died after scouring were obtained as soon as possible after death from the farms in the Edinburgh area on which

they had died. The samples were taken in a similar manner to that described above. The calves involved were either Friesian or Ayrshire, pure or cross-bred. Material was taken from both bull and heifer calves. The average age of the animals was 7 days, and all had scoured for at least 3 days before death. The average time elapsing between death and removal of specimens was 6 hours, and the longest was 10 hours.

After arrival at the laboratory, samples of intestine were opened, washed with iced saline and the excess saline removed with filter paper. They were then sealed between pieces of Parafilm (Gallenkamp, London) and stored at -30°C. Under these circumstances no loss of activity occurred over a period of at least 4 weeks.

Homogenates were prepared by scraping the mucosa with the edge of a microscope slide to take a sample of about 60-80mg (wet weight). The mucosa was then washed into an all glass homogeniser with 2ml of ice-cold saline, and was homogenised by 25 strokes of the plunger. Samples of the homogenate were then removed, diluted by a factor of ten, and assayed for protein and lactase.

Protein was estimated by the method of Lowry, Rosenbrough, Farr and Randall, (1951). Lactase was assayed by the method of Dahlqvist (1964) and was expressed as μMoles of lactose hydrolysed per mg of protein per hour, or, in some experiments, per 10mg wet weight of mucosa per hour.

The optimal substrate pH for calf mucosal lactase was found to be 5.6-5.7 (Fig 27) and therefore the pH used in the assay was 5.65.

The substrate concentration used in the assay of lactase activity was approximately 5x the Michaelis constant for the enzyme. This showed that substrate concentration was not likely to be a limiting factor in the enzyme assay.

It has been found that the mucosal homogenates of some species can inhibit the glucose-oxidase peroxidase system (Asp, Koldovsky and Hoskova, 1967), and it is also possible that mucosal homogenates may remove glucose from the incubation medium by glycolysis. These possibilities were tested by incubating known amounts of glucose with mucosal homogenates in the absence of lactose and then assaying the glucose by the glucose-oxidase method. No glucose loss through glycolysis could be detected, nor was there any inhibition by the homogenate of the enzymes involved in the assay method.

To assess the loss of activity of lactase after death, ligated segments of normal fresh intestine were placed in thermos flasks containing saline at 37°C. Adjacent samples of mucosa were taken initially and at 3 hourly intervals up to 12 hours. Each sample was frozen after removal and was later assayed for lactase activity.

The loss of lactase activity was found to be $2.5 \pm 0.46\%$ per hour($n = 6$). Lactase activity in calves that had been dead for longer than 30 minutes were adjusted accordingly on the assumption that a similar loss occurred in the intact animal after death.

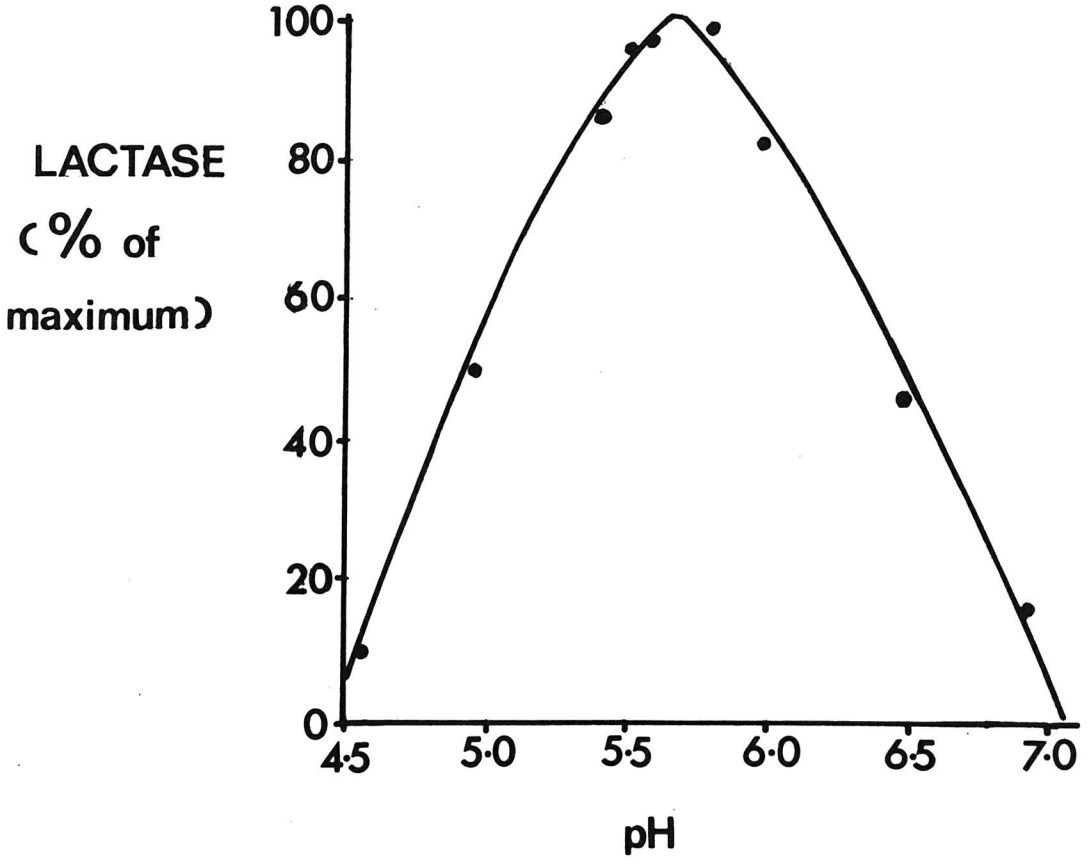


Fig. 27 pH optimum of mucosal lactase from calf small intestine.

(2) The effect of dietary lactose on faeces weight and dry matter in normal calves.

Lactose was added to the milk diet of two normal calves (calf A and B) from the 8th day of life. Faeces were collected in polythene bags fastened by large press studs to a webbing harness. The daily faecal output for each calf was weighed, and a sample was heated to a constant weight to determine dry matter content.

In one animal (calf A) lactose was added to the diet in daily increasing amounts to determine the point at which faeces weight and dry matter content changed. In the case of calf B, a fixed amount of lactose was added to the diet over a longer period. The daily intake of milk and lactose was divided into equal morning and afternoon feeds.

(3) Lactose tolerance tests in calves.

Blood samples were taken from the jugular vein into heparinised evacuated tubes at thirty minute intervals before and after the calf was bucket fed with a measured amount of milk and/or lactose. The samples were deproteinised immediately and frozen until glucose determinations were carried out. Tests were carried out at the same time each day to avoid diurnal variation (Bowen and Reeves, 1967).

Glucose was estimated by the method of King and Wootton 1964, or by the method of Dahlqvist (1964). The latter was found more satisfactory since the chromogen was more stable.

(4) The effect of enterotoxin on lactase activity.

a) In vivo

Samples of mucosa were removed from Thiry-Vella loops in calves using a modified Shiner biopsy capsule (Bywater 1968). Lactase determinations were carried out on these as described earlier. 15ml amounts of enterotoxic culture filtrate were then placed in one of a pair of loops for periods up to 72 hours, with the filtrate replaced by fresh material at 12 hour intervals. Samples were removed for lactase determination before, during and after this period. The other loop of the pair contained saline and was used as a control. Four biopsy samples were taken on each occasion and lactase determinations were carried out on each.

b) In vitro

Since culture filtrates were found to contain lactase activity produced by E.coli, samples were heated at 100°C for 5 minutes to destroy any lactase present.

1 ml of culture filtrate was then mixed with 1 ml of small intestine homogenate, and after incubation for 15 min at 37°C, samples were removed for lactase determination. Uninoculated culture medium was used as a control.

RESULTS.

(1) Lactase activities in healthy calves and calves dying after scour.

Fig.(28) shows the lactase activity in different parts of the small intestine of ten normal calves and ten calves which died after scouring for at least 3 days, (these latter figures have been compensated for post-mortem loss as described above). There were significant differences (Students t-test) between the lactase activities in the two groups at 5% ($p < 0.05$), 25% ($p < 0.01$), and 50% ($p < 0.001$) of the distance between the pylorus and the ileocaecal valve. The differences at 75% and 98% of the distance were not significant ($p > 0.05$ in each case).

In the healthy calves, the maximum lactase activity appeared to lie in the proximal jejunum. The rise between 5% and 25% was not significant, but the fall between 50% and 75% was significant ($p < 0.01$).

(2) The effect of dietary lactose on faeces weight and dry matter content of normal calves.

Fig.(29) shows the effect of the addition of increasing amounts of lactose to the diet of calf A. When the total intake of lactose reached the level of approximately 500g. per day, then the faeces dry matter fell and the total weight rose.

Fig.(30) shows the effect of the addition of a constant amount of lactose to the diet of calf B so that the total lactose intake was approximately 450g. The faeces consistency changed as in calf A, but there appeared to be a

compensation so that the faeces consistency returned toward normal despite a continuing high intake of lactose.

(3) Lactose tolerance tests in normal and scouring calves.

Lactose tolerance tests were carried out on eleven calves, some of which were diarrhoeic. The lactose fed was either 75 or 150 gm (including the estimated lactose content of milk). Most normal calves showed a rise in blood glucose which reached a peak at about 1 hour after feeding (Fig.31).

Some calves showed a flattened lactose tolerance curve, although not all of these were diarrhoeic. Similarly some diarrhoeic calves showed a rise in blood glucose. However when the rise in blood glucose per 25gm of lactose in 36 tolerance tests was plotted against the faecal dry matter percentage (Fig.32) the regression was significant ($p < 0.02$).

(4) The effect of enterotoxic culture filtrates on lactase activity in vivo and in vitro.

a) In vivo

Fig.(33) shows the effect of the presence of enterotoxin in one of two Thiry-Vella loops for periods of 36 and 72 hours. In neither case was there apparent depression of lactase activity in the toxin-containing loop considering the variation in the control loop.

b) In vitro

In four experiments using calf lactase as a substrate, addition of enterotoxic culture filtrate as described caused no depression in lactase when compared with control material.

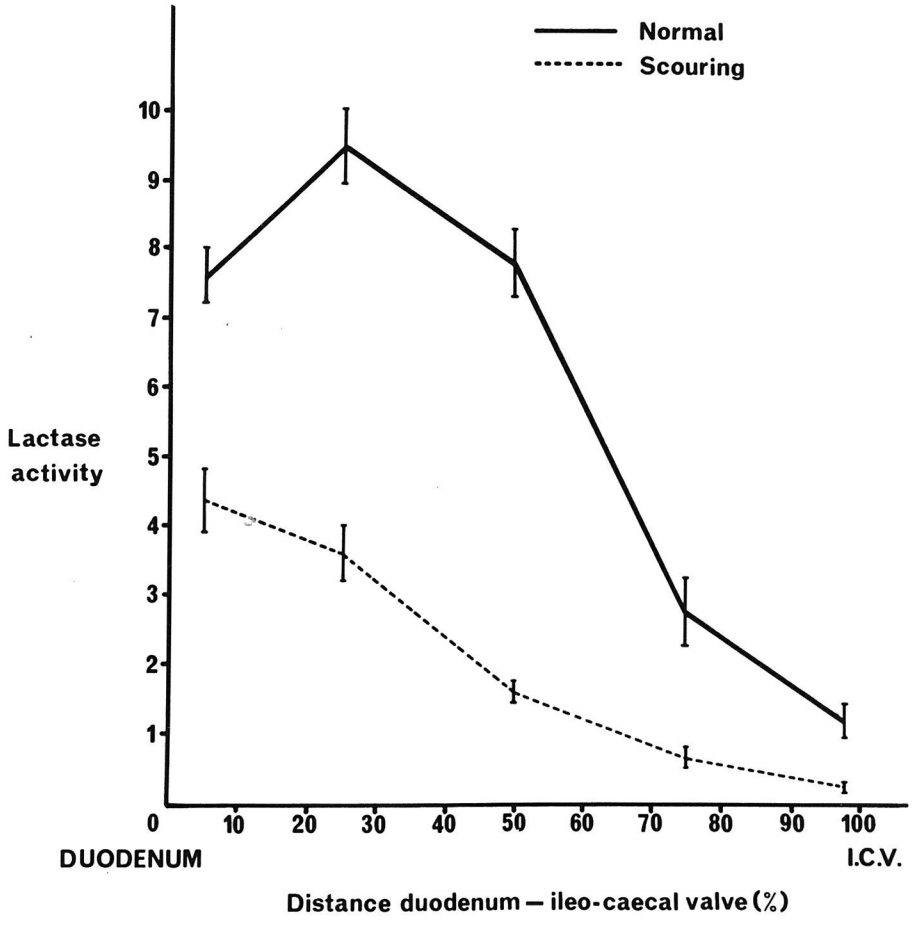


Fig. 28 Lactase activity (μM lactose hydrolysed per mg protein per hr.) in intestinal mucous membrane from healthy and scouring calves. Each point represents the mean of observations on material from 10 animals. Vertical bars show standard errors.

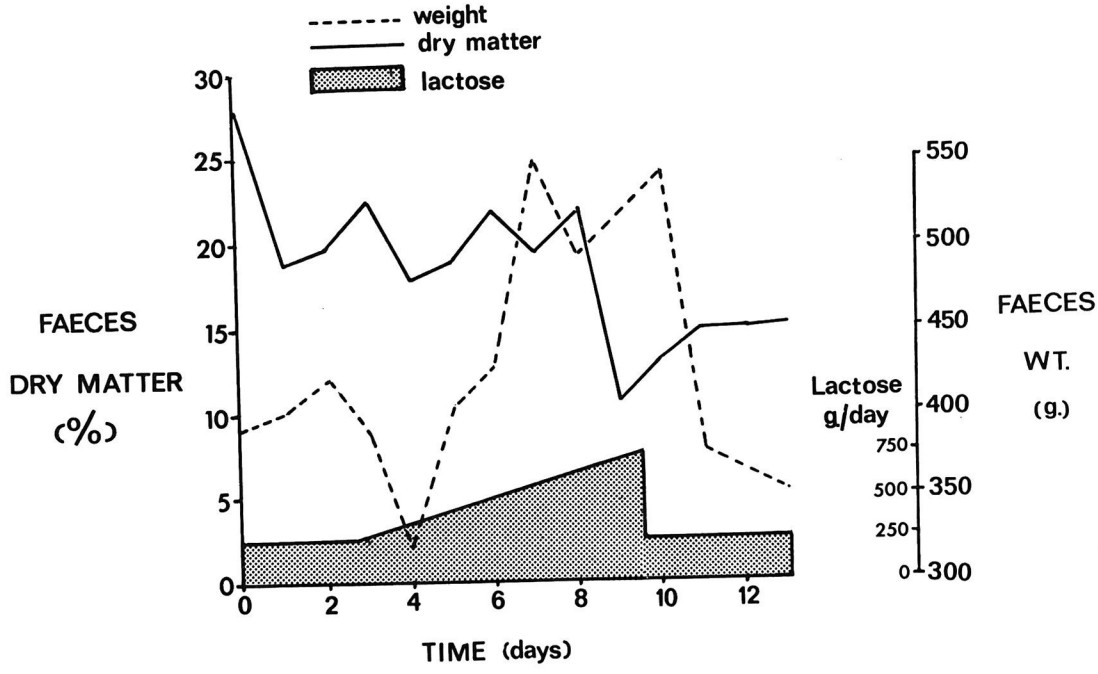


Fig. 29 The effect of increasing dietary lactose on faeces weight and dry-matter in calf A. (10 - 24 days old)

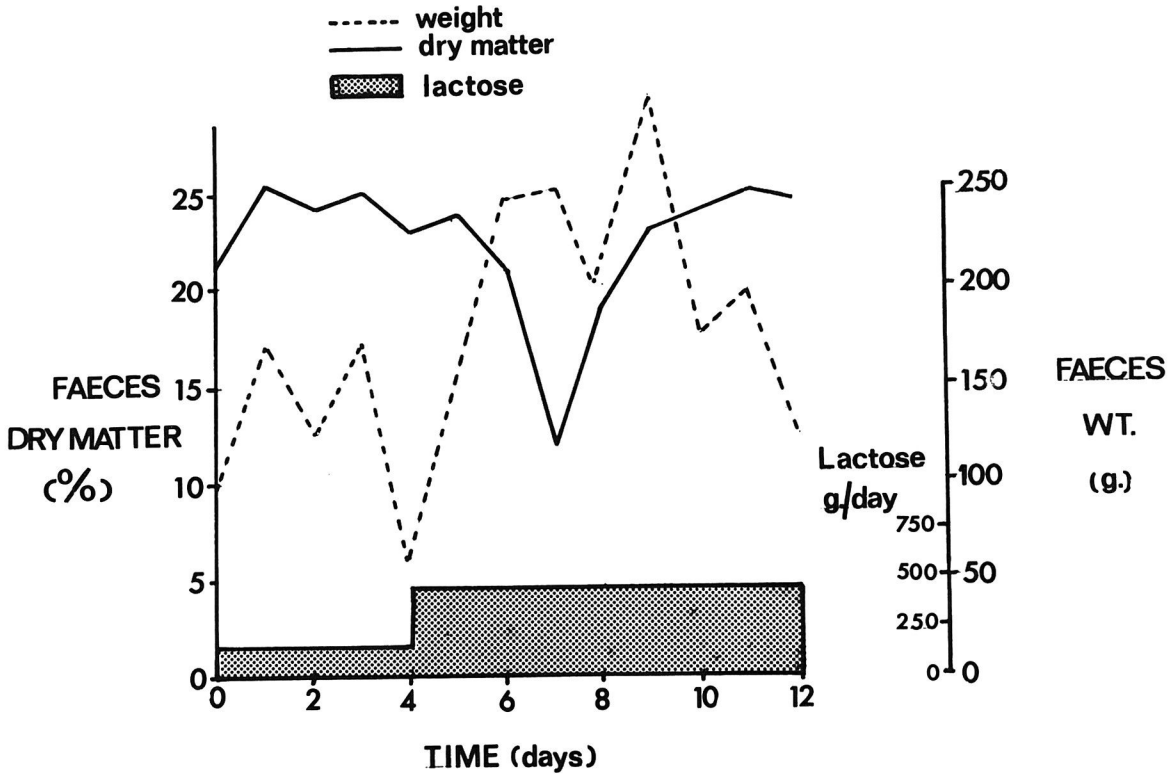


Fig. 30 The effect of increased dietary lactose on faeces weight and dry-matter in calf B. (12 - 24 days old)

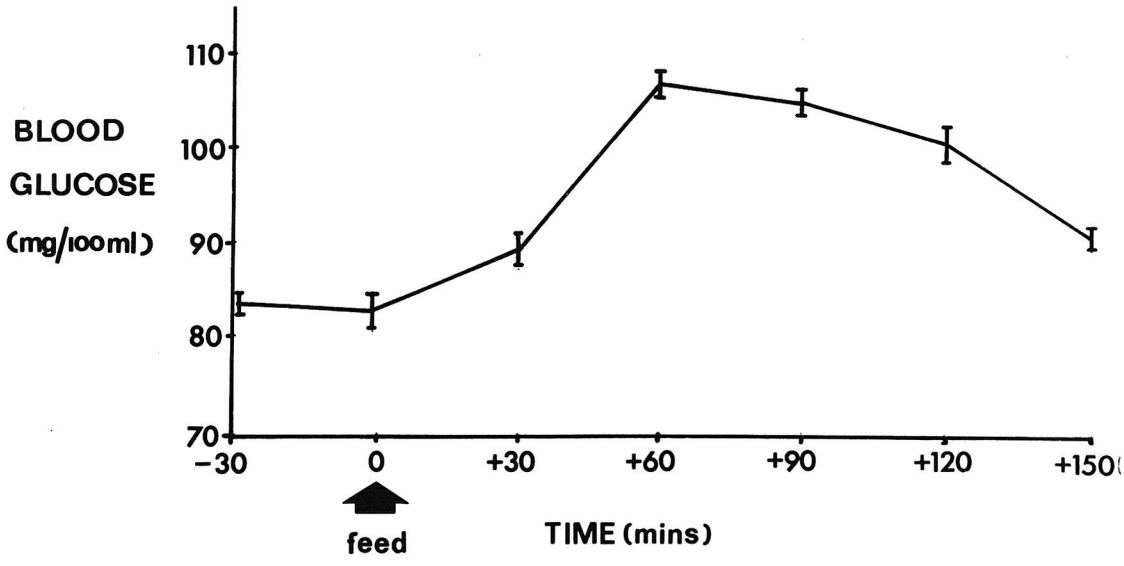


Fig. 31 Blood glucose rise in response to 1.7 l.(3 pints) of milk. Each point represents the mean of at least 10 observations on a single healthy animal between the ages of 10 and 28 days. Vertical bars show standard errors.

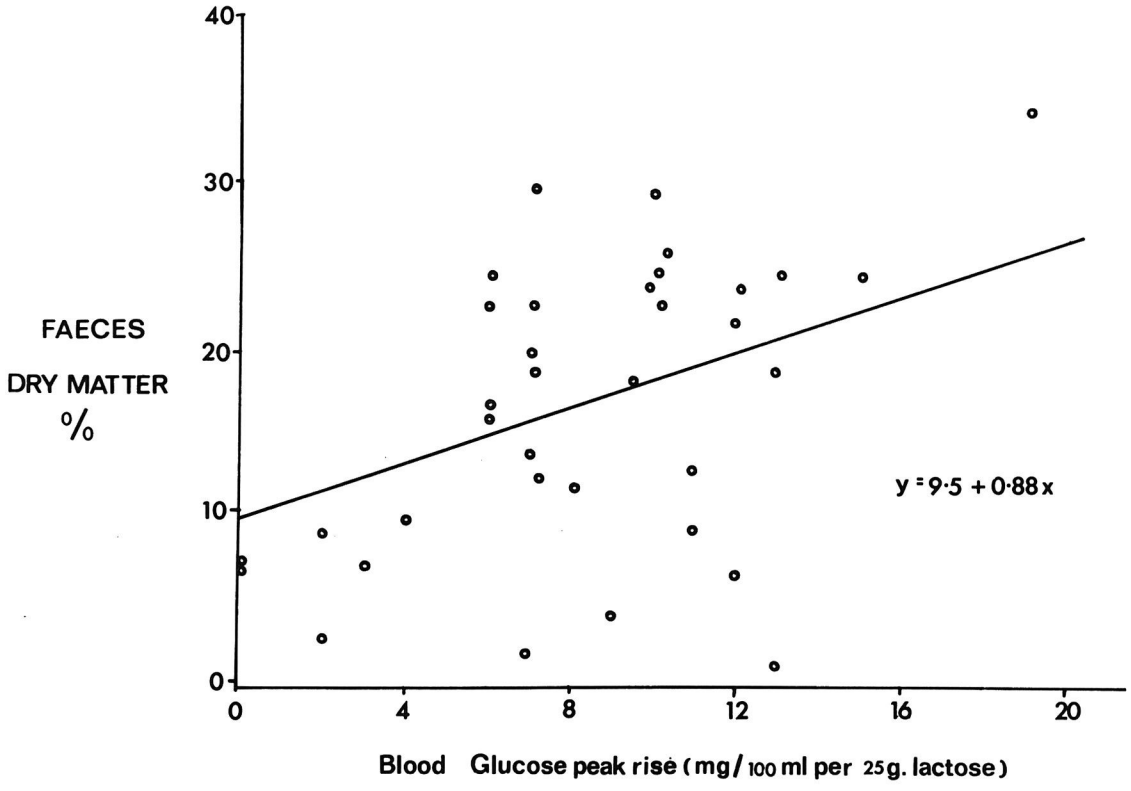


Fig. 32 The relationship between faecal dry-matter content and peak rise of blood glucose. Each point respresents the result of one lactose tolerance test. Observations were obtained from 11 calves, all were less than 3 weeks old, and some remained healthy. The calculated regression line is shown, and regression is significant. ($P < 0.05$).

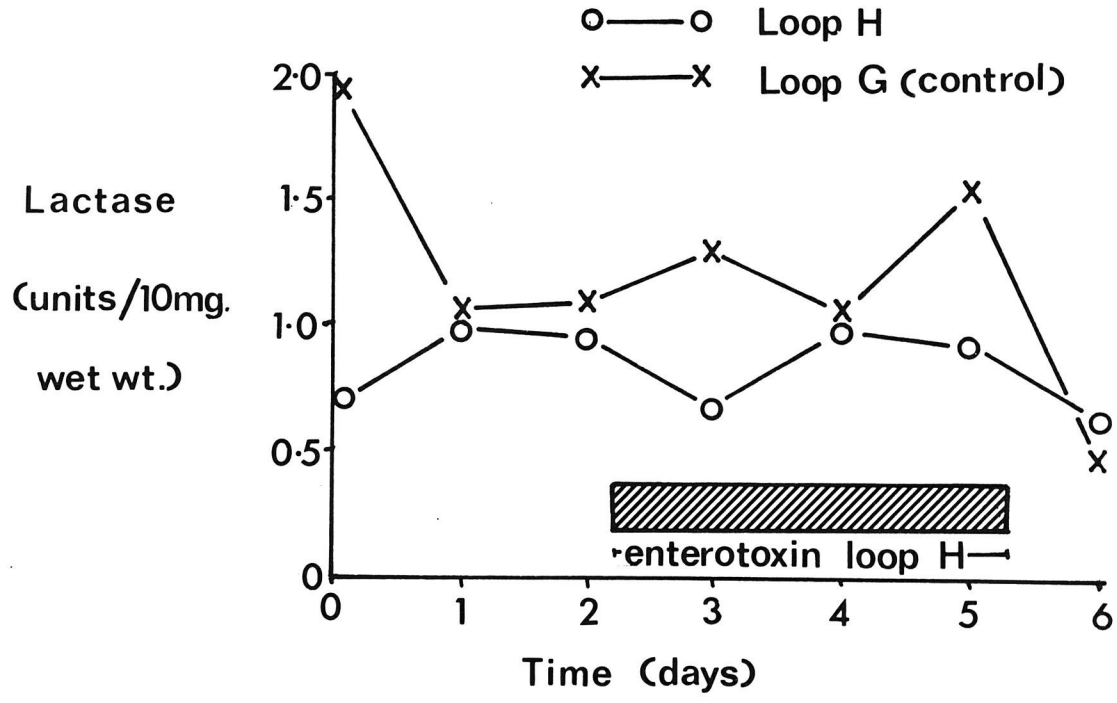


Fig.33 Lactase activity (μM Lactose hydrolysed per 10mg mucosa per hr.) in biopsy specimens from two Thiry-Vella loops (G&H) of upper jejunum.

Each point represents the mean of the results from 4 biopsy specimens.

During the period shown, enterotoxin ($\frac{1}{2}$ standard dose) was placed in loop H, and saline in loop G.

DISCUSSION

The distribution of lactase activity found in normal calves confirms the finding of Siddons (1968) in that highest activities were found in the upper part of small intestine. The distribution also resembled that described by Newcomer & McGill (1966b) in normal human adults.

The depression of activity shown in the calves which died after diarrhoea suggests that these animals may have been unable to digest normally the lactose in their diet, although the relative impediment would depend on the functional reserve of lactase activity.

Blaxter & Wood (1953) showed a progressive decrease in dry matter content of faeces when the lactose & glucose in the diet was raised above 100g/day, although the proportion of lactose was not stated. Rojas et al. (1948) fed calves on a diet containing up to 600g/day of lactose (approximately doubling the lactose intake over usual levels) and found that these animals showed diarrhoea 'within hours'. These authors were unable to show the presence of reducing substances in the faeces of the scouring calves and so suggested that the animals were assimilating the ingested sugar effectively. It seems equally possible however, that microorganisms could have been responsible for the absence of reducing compounds in the faeces.

Mylrea (1966) fed young calves with milk ad lib, and found that they consumed 2.5 to 5.0 l. per meal (the amount taken decreased with time). This amount, which represents 300-500g of lactose per day (2 feeds) was associated with changes in colour of faeces, but not in dry matter content.

The present experiments involving the feeding of lactose to healthy calves show that the two animals tested experienced changes in faecal weight and dry matter content when the total lactose in the diet exceeded about 400g/day. Since the maximal normal lactose intake of a calf below 3 weeks of age is about 150-175g/day, then these animals were receiving approximately 2.5 times the normal intake of lactose before faecal changes were seen. It therefore seems that these healthy animals had an adequate reserve of lactase activity (assuming that added lactose is available for hydrolysis in the same way as milk lactose).

In the case of the animals that died of scour, the depressed lactase level may mean that their reserve capacity was reduced. If the area under the curves in Fig.(28) is taken as a crude measure of the total lactase in the small intestine (and assuming that activities in vivo correspond with those in vitro) the available lactase in the scouring animals appears to be reduced by a factor of 2.8 (i.e. by an amount greater than the apparent reserve capacity of the two normal calves). However a further point needs to be considered since in scouring animals the length of time during which the food material remains in contact with the mucosal enzymes may be reduced. Blaxter and Wood (1953) showed, using marker dye, that fat traversed the whole alimentary tract in 48 hours in normal calves, but in only 6 hours in scouring animals. On the other hand Smith (1964) and Mylrea (1968) both showed, using cannulated calves, that transit time for the ileum alone was not decreased in scouring animals. However they found that the volume of the ileal effluent was increased, so that dilution of luminal contents may decrease the contact of lactose with mucosal lactase even if transit time is unimpaired.

Undigested lactose reaching the large intestine would be likely to produce a fermentative (Weijers and Van der Kramer, 1963) or osmotic (Christopher and Bayless, 1968) diarrhoea.

The transitory nature of the change in faecal consistency in calf B despite a continued high intake of lactose may have been an example of induction of intestinal lactase by a high lactose diet. This phenomenon has been demonstrated in rats (Girardet, Richterich and Antener, 1964; Cain, Moore, Patterson and McElveen, 1969). Alternatively the temporary nature of the change may have reflected a readjustment of the bacterial flora.

The lactose tolerance tests carried out on normal and scouring calves showed that diarrhoeal faeces were associated with a lower peak of blood glucose after lactose feeding. This can be explained in more than one way:

a) A mucosal lactase deficiency would produce such a change since less glucose would be formed and undigested lactose might then be responsible for further osmotic or fermentative diarrhoea.

b) If lactase activity were normal, then as previously mentioned, either increased rate of passage, or dilution of luminal contents may decrease the contact time between the mucosal lactase and the lactose in the gut lumen.

c) If lactose hydrolysis were normal, the flattened tolerance curve might also be explained by a glucose absorption defect. Glucose malabsorption has been found in acute infant diarrhoea (Rodriguez-de-Curet, Lugo-de-Rivera and Torres-Pinedo, 1970) but these workers found that the glucose absorption defect, although significant, was minimal in comparison with the defect in lactose hydrolysis.

Glucose malabsorption seems a relatively improbable explanation, especially since animals with flat lactose tolerance curves responded to glucose feeding by a normal rise in blood glucose. The results of mucosal lactase determinations (Fig 28) appear to provide a more likely explanation for the flattening of the lactose tolerance curve, although rapid movement of gut contents may also contribute to the effect.

The use of lactose tolerance tests in man has been criticized as giving only limited information about mucosal lactase activity, and it has been suggested that delayed gastric emptying may give flattened tolerance curves in subjects with normal mucosal lactase activities (Newcomer and McGill, 1966a). Other authors however, have found good correlation between depressed blood glucose responses in lactose tolerance tests and low lactase activity in mucosal biopsies (Peternel, 1965; Welsh, 1966).

It seems likely that the rather scattered nature of the results in Fig(32) may result both from the limitations of the lactose tolerance tests as a means of assessing mucosal lactase activity and also from the fact that the peak of blood glucose activity is only an approximate indicator of total glucose absorption. The area beneath the blood glucose curve might have been a more accurate indicator of the total glucose absorbed, but appeared to be equally subject to variation and so was not used in place of peak height.

The present results do not indicate whether the lactase deficiencies seen in the animals dying of scour were primary (congenital) or secondary to mucosal damage associated with diarrhoea. While a few cases of primary lactase deficiency have been reported in Poland (Kwiatkowski, 1967) complete alactasia

seems likely to be a relatively rare phenomenon in calves.

Secondary lactase deficiency has also been reported by Kwiatkowski (1967) in calves with 'digestive tract necrosis' and by several authors in human patients with enteritis (Haworth and Ford, 1960; Careddu, Giovannini and Cevini, 1963; Sunshine and Kretchmer, 1964; Hirschhorn and Molla, 1969). In this case the deficiency appears to be the result of mucosal damage, or possibly of increased rate of migration of villous epithelial cells. A secondary lactase deficiency appears to be a likely explanation for the findings among scouring calves in the present experiments. It was also noted that two of the group of ten 'normal' calves showed mucosal lactase activity little greater than the mean of the scouring animals. These animals may have been particularly prone to further lowering of enzyme activity by mucosal damage.

Enterotoxic culture filtrates did not appear to have any inhibitory capacity for lactase activity in the experimental conditions under which it was tested. If associated with fluid loss and diarrhoea, enterotoxin might be expected to have an indirect effect on lactose hydrolysis by reducing contact time between luminal contents and the mucosal enzyme, and also by diluting lumenal contents and so further reducing such contact.

The depression of lactase activity seen in the calves examined may provide some basis for the common practice of reducing milk intake of scouring calves (Roy, 1970).

GENERAL DISCUSSION

The initial experiments confirmed that enterotoxic culture filtrates caused fluid accumulation in ligated loops of calf intestine. Moreover, the failure to demonstrate activity in other experimental systems suggested that the effect was possibly specific for the intestine of the calf.

For this reason, Thiry-Vella loops were examined as a possible test preparation, and were found to be suitable for the study of E.coli enterotoxin in calves. This approach had advantages over the ligated loop technique in that it allowed replication of experiments in the same loop of intestine, it also allowed close monitoring of fluid and electrolyte changes in the lumen, and was economical in the use of experimental animals.

The results of experiments to determine the effect of enterotoxic extract on net movement of fluid and solutes showed that it caused a net shift towards secretion of fluid, sodium, bicarbonate and chloride. Potassium was affected inconsistently, and glucose absorption was unchanged. A similarity was noted between these findings and those of other workers using V.cholerae enterotoxin in other species.

It appeared that a study of the effect of E.coli enterotoxin on unidirectional fluxes of fluid and sodium might provide useful information on the mode of action of enterotoxin on transport mechanisms in the small intestine. For these experiments, it was necessary to use deuterium oxide and sodium-22 as isotopic labels, assuming that they would be treated in the same way as water and sodium. However, in preliminary experiments using rat intestine in vitro, it was shown that an isotope effect of deuterium occurred in this preparation,

but it was hoped that, if a similar effect occurred in the calf, it might have relatively little effect on the qualitative nature of the results obtained.

The effect of E.coli enterotoxin on the unidirectional fluxes of fluid and sodium appeared to be complex, since the net effect on fluid movement was the result of an increase in exsorption, while the net effect on sodium movement was mainly the result of a decrease in insorption. These results appeared to suggest therefore, that the unidirectional fluxes of water and sodium were largely independent, despite an apparant relationship between the net movement of fluid and sodium. It was possible to give only a speculative explanation of mechanisms which may have been involved in the activity of enterotoxin.

The examination of a series of strains of E.coli from healthy calves and also from scouring and septicaemic animals indicated that the strains from scouring calves were potent producers of enterotoxin as judged by the effect on water absorption from Thiry-Vella loops. However, commensal strains also appeared to produce some effect on absorption, which suggested that enterotoxin production was a graded phenomenon. Nevertheless, the results suggested that enterotoxin production was an important factor in outbreaks of scour in calves.

The experiments carried out in an attampt to characterise the enterotoxic material showed that it was distinct from bacterial endotoxin. It was also found to be dialysable, and ultrafiltration experiments showed that its molecular weight lay mainly between 1000 and 10,000. The heat stability was confirmed, and a dose-response relationship was shown to exist using freeze-dried dialysate material.

Experiments to establish whether systemic absorption of enterotoxin was occurring were not conclusive, since a positive result in one animal could not be confirmed in a second animal. If present, systemic absorption of enterotoxin may mean that enterotoxin-producing organisms in the large intestine could affect fluid and electrolyte transport mechanisms in the upper intestine.

The experiments carried out to determine lactase activity in intestinal mucosa of healthy calves and those which had died after scouring showed that activity was depressed in the latter group. However, healthy calves were shown to possess some reserve of lactase activity, since the dietary lactose needed to be more than doubled in order to affect the dry-matter content of the faeces.

On the other hand, lactose tolerance tests in healthy and scouring calves showed that a correlation existed between the maximum rise in blood glucose and the faecal dry matter content. This suggested that the lactose was not being fully utilised by the scouring animals, and that the fermentative effects produced by milk feeding may aggravate existing diarrhoea.

No link could be demonstrated between enterotoxin and lactase activities, suggesting that some other mechanism was responsible for the low lactase activities seen in calves which had scoured.

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APPENDIX 1.

CALCULATION OF NET MOVEMENT OF FLUID AND SOLUTES FROM THIRY-VELLA LOOPS

a) Fluid

Let PEG concentration in test sol = a mg/ml.

" " " in the sample taken after mixing with residual fluid = b mg/ml

" " " after 30 mins. = c mg/ml.

If 30ml is the volume of solution placed in the loop.

The Fluid absorbed = $(\frac{a}{b} \times 30) - (\frac{a}{c} \times 30)$ ml per 30 min.

$= 30a(\frac{1}{b} - \frac{1}{c})$ ml per 30 min.

b) Potassium, bicarbonate and glucose

The calculation for movement of potassium, bicarbonate and glucose is similar.

e.g. for potassium.

Let the concentration of potassium in test solution be K1 m.eq/l.

" " " " " after 30 min. be K2 m.eq/l

Then if 30 ml. is the volume placed in the loop

Potassium absorbed = $0.030 (K1 - \frac{a}{c} \times K2)$ m.eq/30 min.

c) Sodium and chloride

For these ions, allowance is made for the residual saline in the loops at the start of the 30 minute absorption period, assuming that its sodium concentration will be 160 m.eq/l. The length of time between draining of saline and placing of test solution in the loop was minimised to support this assumption.

e.g. for sodium:-

Amount of sodium in residual saline at start of 30 minute period

$$= .03 \left(\frac{a}{b} - 1\right) 160$$

$$= 4.8 \left(\frac{a}{b} - 1\right) \text{meq.}$$

Let the concentration of sodium in test solution be Nal m.eq/l

" " " " " after 30 min. be Na2 m.eq/l

If 30 ml. is the volume of test solution placed in loop.

Then sodium absorbed = $\dfrac{4.8\left(\frac{a}{b} - 1\right) + 0.030 \left(\text{Nal} - \frac{a}{c}\,\text{Na2}\right)}{\text{m.eq/30 min.}}$

APPENDIX 2

CALCULATION OF UNIDIRECTIONAL MOVEMENTS OF FLUID AND SODIUM TO AND FROM THIRY-VELLA LOOPS.

The unidirectional movement of fluid or electrolyte may be calculated from the disappearance of the isotopic tracer using the formula of Visscher, Varco, Carr, Dean and Erickson (1944). The formula given by these workers may be expressed:

$$\text{Insorption} = \frac{\text{Quantity of isotope lost from lumen}}{\text{Arithmetic mean of the specific activity of the lumenal solution.}}$$

Since Net movement = Insorption - Exsorption

Then exsorption may be calculated from a knowledge of insorption and net movement.

This method has been criticized by Berger and Steele (1958) since the mean specific activity is not the arithmetic mean, but rather a function of (1) the integral of the change in specific activity and (2) the effects of the net change. These authors therefore suggested a formula allowing for this, but noted that with changes in specific activity of less than 40%, there is little difference between the arithmetic and true means. With larger changes in specific activity, the arithmetic mean overestimates the true mean at a rapidly increasing rate.

In the present experiments, the change in the specific activity of sodium$_{22}$ was consistently less than 40%, and so the above formula was used. The change in the specific activity of the deuterium oxide was, however, greater than 40% therefore the formula given by Berger and Steele (1958) was used.

$$\text{Insorption} = \frac{A - Ao}{t} \left[\frac{\ln \frac{(SA - SB)}{(SAo - SBo)} + \ln \frac{B}{Bo}}{\ln \frac{B}{Bo} - \ln \frac{A}{Ao}} \right] -1$$

Where:

Ao = total amount of labelled & unlabelled material in lumen at time 0

A = " " " " " " " " " " t

Bo = " " " " " " " plasma " " 0

B = " " " " " " " " " " t

SAo= specific activity of D_2O in lumen at time 0

SA = " " " " " " " " t

SBo= " " " " " plasma " " 0

SB = " " " " " " " " t

In the present experiments B_o and B are large in comparison with the change, and if the assumption is made that the change is negligible, the equation becomes:

$$\text{Insorption} = \frac{A - Ao}{t} \left[\frac{\ln \dfrac{(SA - SB)}{(SAo - SBo)}}{-\ln \dfrac{A}{Ao}} \right] -1$$

But if SB and SBo are small,

$$\text{then Insorption} = \frac{A - Ao}{t} \left[\frac{\ln \dfrac{SA}{SAo}}{-\ln \dfrac{A}{Ao}} \right] -1$$

This formula is the one used to calculate the unidirectional movement of fluid.

APPENDIX 3.

EXPERIMENTS TO TEST THE VALIDITY OF DEUTERIUM OXIDE AS AN ISOTOPIC LABEL FOR THE STUDY OF WATER MOVEMENT.

Introduction.

Deuterium oxide has been used as an isotopic label by a number of authors to follow the unidirectional fluxes of fluid to and from the intestinal lumen (Visscher,Fetcher, Carr, Gregor, Bushey and Barker,1944; Grim, 1962; Swallow, Code and Freter, 1968). In each case, the assumption has been made, either tacitly or explicitly, that the isotopic effect of deuterium is negligible, so that its movement can be taken as an indicator of fluid fluxes.

The evidence in the literature concerning the diffusion rates of D_2O, HDO and H_2O is not clear. Hevesy, Hofer and Krogh (1935) found that the rate of diffusion of water across frog skin was more rapid than the movement of deuterium oxide; Parpart (1935) and Brooks (1935) both found that haemolysis of erythrocytes occurred more slowly in deuterium oxide than in water; the former author showed that a 75% deuterium oxide solution caused a 25% decrease in the rate of haemolysis. Observations on the rate of diffusion of deuterium oxide into protozoa (Kitching and Padfield, 1960) suggested that the rate of penetration of deuterium oxide might be 10% less than that of water.

On the other hand, the rate of penetration of deuterium oxide into sea-urchin eggs (Lucke and Harvey, 1934) and into giant amoeba (Lovtrup and Pigon, 1951) could not be differentiated from that of water.

The rates of diffusion of deuterated and tritiated water have been compared in different preparations, and have either been found to be indistinguishable (Chinard and Enns, 1954; Takashina, Lazzara, Cronovich and Burch, 1962; King 1969) or the differences seen have been attributed to experimental artifacts such as unstirred layers (Elford 1970). This has been taken as indirect evidence for the validity of studies using hydrogen isotopes as markers for water transfer (King 1969).

The absence of an isotope effect in deuterium oxide movement during intestinal movement cannot therefore be said to be resolved. The present experiments were designed to show whether an isotope effect existed in the transport of deuterium oxide by rat intestine *in vitro*.

Materials and Methods.

These experiments were carried out using the *in vitro* perfusion method of Fisher and Gardner (1971) whose help is gratefully acknowledged.

The small intestine was removed from etherised rats, and the lumen was perfused with a segmented stream of modified Krebs Hensleit (plus glucose) solution separated by oxygen bubbles. The intestine was maintained in a moist, warm environment, and remained viable for some hours (Fisher and Gardner,1971). Absorbed material falls from the serosal surface and may be collected.

About five minutes after the intestine was set up the perfusate was changed for a similar one containing deuterium oxide (1% w/v nominal), and after allowing time for the lumen perfusate to reach a steady state, the serosal secretion was collected at 2 minute intervals.

The samples of serosal secretion were weighed, diluted with 0.5ml water and centrifuged. Deuterium oxide in the serosal samples and in the perfusate was estimated by infra-red absorption as described earlier.

The concentration of deuterium oxide in the serosal samples rose exponentially. A digital computer programme kindly supplied by Dr.G.Atkins was used to fit a function of the following form to the data.

$$C = B + A. \exp(-kv)$$

Where C is the concentration of D_2O in a sample of secretion

v is the cumulated volume of secretion to the mid-point of the collection (i.e. the sum of all the volumes collected up to the current collection, plus half the volume of the current collection).

and B, A and k are constants.

As shown in the following section, the constant term B represents the deuterium oxide concentration in the secretion at the steady state. If there were no isotope effect, this concentration (B) would be indistinguishable from the concentration of deuterium oxide in the luminal perfusate.

Theoretical analysis of the kinetics of deuterium oxide absorption. (Prepared by Professor R.B.Fisher, whose help is gratefully acknowledged).

Consider the time course of transport of deuterated water from the lumen of the intestine via the extracellular fluid and to the serosal surface of the intestine.

Suppose that deuterium oxide and water are absorbed at different rates so that the concentration of deuterium oxide in the insorbed fluid is different from that in the lumen. The concentration of the marker in exorbed fluid also differs from the concentration in the extracellular fluid.

Let:- P be the luminal concentration of deuterium oxide (supposed constant)

C " " concentration of deuterium oxide in the extracellular fluid, and hence in the secretion.

i be the rate of insorption of water.

e be the rate of exsorption " "

s " " " " fluid secretion at the serosal surface.

V be the volume of extracellular fluid (supposed constant).

$$a = \frac{\text{Deuterium oxide concn. in insorbed fluid}}{\text{Deuterium oxide concn. in lumen.}}$$

and

$$b = \frac{\text{Deuterium oxide concn, in exsorbed fluid.}}{\text{Deuterium oxide concn. in extracellular fluid.}}$$

v = Cumulated secretion.

B = steady state concentration of deuterium oxide in serosal secretion.

$$r = \frac{i}{s}$$

since V is constant, $i = e + s$.

So, $e = (r-1)s$

With very small changes in i, e and s, the corresponding change in C is given by:-

$$V.dc. = aP.di. - b.c.de. - C.ds.$$

$$\frac{dc}{\frac{a\,Pr}{b(r-1)+1} - C} = \frac{b(r-1)+1}{V}.ds$$

On integration, this yields an exponential function of the form:

$$C = B + A.\exp(-kv)$$

where $B = \frac{aPr}{b(r-1)+1}$ = the value of C at the steady state

$A = Co - B$ where Co is the value of C when $v = 0$

k (the rate constant) $= \frac{b(r-1)+1}{V}$

and v is the cumulated volume secreted.

If there is no isotope effect,

$a = b = 1$

Then B = C that is the concentration of the marker at the steady state which will equal the concentration in the lumen.

Measurement of C at successive values of v will allow estimation of B and k.

If the isotope effects for the insorption and exsorption are equal (a=b) then the terms for B and k can be solved for b.

and then $b = 1 - V.k\left(1 - \frac{B}{P}\right)$

Results.

Mean values of the relevant parameters from 11 experiments are shown below, together with their standard errors.

Perfusate concentration (P) = 1.123 ± 0.012

Computed steady state serosal concentration (B) = 1.0966±0.0134.

Rate constant (k) = 0.1605±0.024

If a = b

$$b = 1-Vk\left(1 - \frac{B}{P}\right)$$

The volume V may be taken from other experiments (Fisher & Gardner 1971) to be 30.9µl/cm.

then a = b = 0.891±0.027.

If there were no isotope effect, then a = b = 1. The observed effect is significant ($P < 0.01$, paired t-test).

Discussion

The present experiments appear to have considerable relevance for the use of deuterium oxide as a marker in intestinal absorption studies, and the discovery of an 11% difference in the concentration of deuterium in insorbed and luminal fluid indicated that a significant isotope effect was present. The figures obtained assume that isotope effects in insorbed and exsorbed fluid are equal (i.e. $a = b$) but even if $a \neq b$ an isotope effect will still exist.

The implications of the findings, if true for deuterium oxide movement from the intestine of other species, is that the calculated values for insorption and exsorption obtained from measurement of disappearance of the isotope from the lumen will tend to be underestimated.

Thus the values for fluid fluxes in the experiments using deuterium oxide in calf Thiry-Vella loops may be greater than suggested, assuming that a similar isotope effect exists under these conditions. However, it does not seem justifiable to transfer the findings quantitatively from an experiment *in vitro* in one species, to one *in vivo* in another.

The effect of an isotope effect of this magnitude on the results of experiments involving enterotoxin activity will be negligible in those experiments where fluid insorption was unaffected, but exsorption was increased. Where enterotoxin was found to alter fluid insorption, then the magnitude of this change will probably be underestimated as a result of the isotope effect. The qualitative effect of enterotoxin should, however, be unchanged.

APPENDIX 4.

PUBLISHED PAPERS

SOME EFFECTS OF *ESCHERICHIA COLI* ENTEROTOXIN ON NET FLUID, GLUCOSE AND ELECTROLYTE TRANSFER IN CALF SMALL INTESTINE

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INTRODUCTION

It has been shown that living cultures of certain enteropathogenic strains of *Escherichia coli* can cause dilation when injected into ligated loops of intestine, while non-enteropathogenic strains do not (Taylor, Wilkins and Payne, 1961; Gyles and Barnum, 1967; Smith and Halls, 1967a). Smith and Halls (1967b) also showed that dilation could be caused by sterile filtrates of soft-agar cultures of enteropathogenic strains of *E. coli*, or by acetone extracts prepared from these filtrates. They suggested, therefore, the presence of an "enterotoxin" which they showed to be heat-stable and non-antigenic. Gyles and Barnum (1969) used a different method to prepare an enterotoxin from enteropathogenic strains of *E. coli* which they showed to be heat-labile and antigenic. Nevertheless, it has been suggested (Smith and Gyles, 1969) that these two enterotoxins are probably different forms of the same entity.

Ligated loops studies, while adequate for qualitative examination of enterotoxin activity, have certain limitations; for example they are poorly suited to the study of the recovery of loops after enterotoxin activity and are expensive in terms of experimental animals. In the present experiments therefore, Thiry-Vella loops were prepared in calves to allow replication in the same preparation, and to permit the study of the effect of enterotoxin on absorption of fluid, electrolytes and glucose.

MATERIALS AND METHODS

Enterotoxin preparation. The organism used for production of enterotoxin was the *E. coli* designated B44 (untypable) kindly supplied by Dr. H. Williams Smith. Preliminary experiments had confirmed that soft-agar culture filtrates of this organism caused dilation of ligated loops in calves. Culture filtrates were prepared as described by Smith and Halls (1967b) except that neomycin was omitted. Standard amounts of acetone extract were made by adding 8 vol. of acetone to 30 ml. soft-agar culture filtrates, and allowing them to stand overnight at −30°C. Uninoculated culture medium was treated in an identical manner and used as a control. Acetone extracts were dried and redissolved in 30 ml. of test solution I.

Test solution constituents. Test solution I contained NaCl (49·56 m eq/l.), $NaHCO_3$ (26·0 m eq/l.), KCl (4·0 m eq/l.), glucose (10·0 g./l. except for loops A and B, where glucose concentration was 1·0 g./l.) and polyethylene glycol as a marker (PEG 7·5

g./l.). After addition of acetone extract, this solution was approximately isotonic. In experiments where no acetone extract was added, a second test solution (II) was used in which the NaCl content was raised to 97·72 m eq/l. to maintain approximate isotonicity.

Surgical procedures. Two adjacent Thiry-Vella loops approximately 25 cm. in length were prepared in Ayrshire bull calves under cyclopropane anaesthesia at 1 to 3 weeks of age. Pairs of loops were prepared in the upper jejunum of two calves about 3·5 m. from the pylorus and were designated E and F (first calf) and G and H (second calf). Further pairs of loops were prepared in two other calves from the lower ileum about 2 m. from the ileo-caecal valve. These were designated A and B, and I and J, respectively. Loops were not used for absorption studies for three weeks after preparation, and were washed at least once every two days with isotonic saline at 37°C. to remove cellular debris.

The ends of the loops were sealed by Foley catheters (female 18Fr.) fitted with 10 cm. perforated extensions which lay in the lumen of the loops. A foam rubber, latex covered cone fitted over each catheter and lay on the outside of the stoma holding the inflated balloon snugly against the inside, and ensuring that the catheter was maintained at right-angles to the abdominal wall. The ends of the catheter were closed by spring clips.

Experimental procedures. The absorption of fluid, glucose and electrolyte was observed during two consecutive 30 minute periods in each loop. During the first 30 minutes the control solution was used (test solution I plus acetone extract from 30 ml. uninoculated soft-agar filtrate) while during the second 30 minutes test solution I plus acetone extract from *E. coli* culture filtrate was used. In this way each loop was used as its own control. Absorption from identical solutions (solution I plus control extract, or solution II alone) did not differ significantly between consecutive 30 minute periods.

Before each experiment, with the animal standing quietly in a holding crate, the catheters were placed in position and the balloons inflated. The loops were irrigated with warm isotonic saline until the washings were clear of mucus and debris. The catheters were clamped, and 30 ml. of the test solution containing control extract at 37°C. was placed in the loop. After mixing thoroughly with the residual saline a 0·1 ml. sample was removed for PEG estimation to determine this residual volume. After 30 minutes, the loops were drained and washed thoroughly with warm saline before the second 30 minute period during which the test solution I and enterotoxin extract was used in a similar manner. The fluid recovered after each test period was sampled for PEG, electrolyte and glucose analysis. Leakage from the loops was rare, but where it occurred the results from that experiment were discarded.

Recovery of normal fluid absorption after enterotoxic activity was followed by measuring the absorption of fluid from test solution II before, and at intervals after, exposure to the standard amount of enterotoxin for 30 minutes.

The toxicity of the culture fluid extract for mice was determined by the use of a concentrated solution of acetone extract from *E. coli* culture fluids obtained from 300 ml. of culture fluid, which was dissolved in 20 ml. of sterile distilled water. The solution was injected intraperitoneally into groups of mice, and the LD_{50} was calculated using the method of Reed and Muench (1938). The LD_{50} for phenol-water extracted endotoxin was determined in a similar manner.

Biochemical procedures. PEG was estimated by the turbidometric method of Hyden (1956) except that turbidity was observed after exactly 60 minutes (Smith, 1959) at 650 mμ in an EEL spectrophotometer.

Sodium and potassium were estimated by flame photometry (EEL flame photometer) bicarbonate by the microdiffusion method (Conway, 1962), glucose by the glucose oxidase dianisidine method (Dahlqvist, 1964) and chloride by the EEL chloridemeter. The change in concentration of PEG was used to calculate fluid transfer, and the calculations of electrolyte and glucose transfer were adjusted accordingly. The

sodium and chloride values were also adjusted to allow for the residual saline in the loop at the start of the test period.

Osmolality was determined cryoscopically using a modification of the Hortvet apparatus. Bacterial lipopolysaccharide (endotoxin) was prepared from the same strain of organism by phenol-water extraction (Westphal, Lüderitz, Eichenberger and Keiderling, 1952).

RESULTS

Preliminary experiments showed a mean recovery for PEG of 97 per cent. showing that the substance was not significantly absorbed during the period of study, and confirming the finding of Smith (1962). Osmolality of the control solution (sol. I plus acetone extract of uninoculated soft-agar filtrate) was 322 ± 6 mosm/kg. while osmolality of the enterotoxin containing solution was 328 ± 11 mosm/kg. These do not differ significantly from one another (Student's t-test).

Effect of Enterotoxin

Fluid movement. In each of eight loops examined (4 calves) the presence of enterotoxin caused a significant shift towards net secretion in comparison with the preceding control period (Fig. 1a).

Electrolyte movement. A shift towards secretion was seen in the net transfer of sodium (8 loops examined, Fig. 1b), bicarbonate (4 loops examined Fig. 2) and chloride (2 loops examined Fig. 2). The effect on potassium was inconsistent, being significant in only 2 of the 8 loops examined (Table 1).

Glucose movement. There was no effect of enterotoxin on glucose movement in any of the eight loops examined (Table 1).

Fluid electrolyte and glucose absorption. The addition of endotoxin (5 mg./30 ml.) to test solution II had no effect on net transfer of fluid, sodium, potassium, bicarbonate or glucose in either of two loops examined (Table 2).

Mouse Toxicity of Endotoxin and Enterotoxic Extract

The LD_{50} for B44 endotoxin prepared by phenol-water extraction was 1·3 mg. for 20 g. mice. The LD_{50} for acetone extract from culture fluids of *E. coli* was equivalent to 19 ml. of the culture filtrate. In this case 30 ml. of culture filtrate probably contained not more than 2 mg. of endotoxin.

Recovery of Loops after Enterotoxic Activity

When absorption of fluid from test solution II was examined in loops I and J before and after a 30 minute exposure to the standard amount of enterotoxic extract, recovery appeared virtually complete within five hours (Figs. 3a, 3b).

DISCUSSION

These experiments show that Thiry-Vella loops may be used in calves to demonstrate absorptive changes in response to *E. coli* enterotoxin. The method allows replication in the same animal on different occasions, relating fluid and

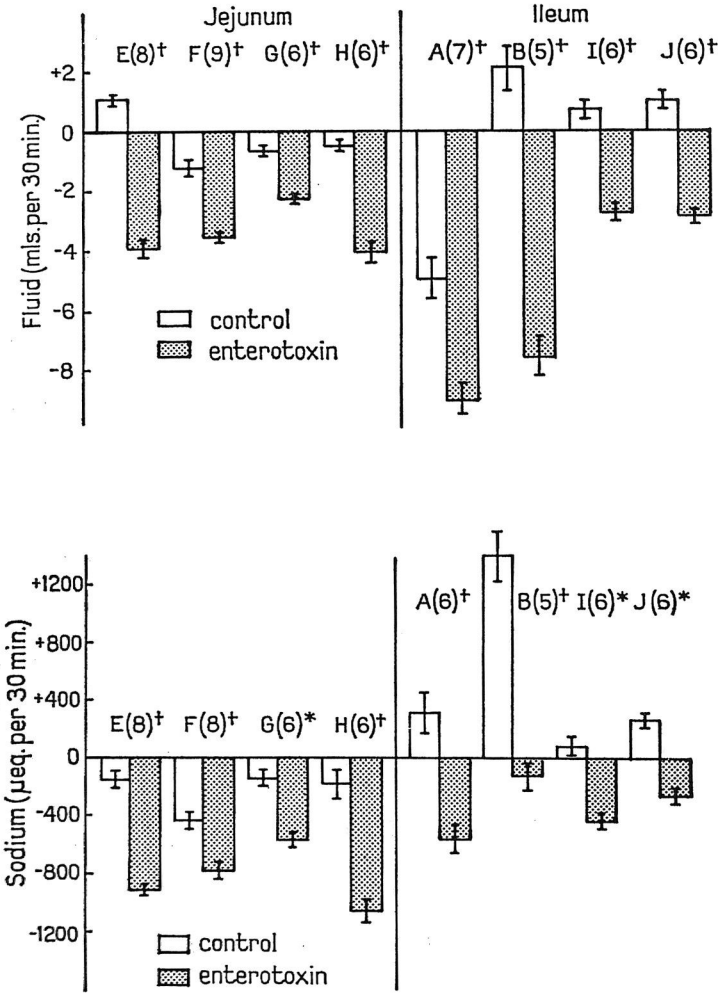

Fig. 1. The effect of enterotoxin on fluid (a) and sodium (b) absorption in four jejunal loops (E, F, G & H) and four ileal loops (A, B, I & J). Each column represents the mean of the number of observations given in parentheses. Positive signs indicate net absorption, negative signs indicate net secretion. Vertical bars represent S.E. Differences are significant in each case, (Student's t-test $p < 0{\cdot}05$ [*] or $p < 0{\cdot}01$ [†]).

electrolyte changes to time and examination of recovery after enterotoxic activity; it is also economical in experimental animals. It therefore has advantages over the ligated loop technique.

The results show that the onset of the effect of the enterotoxin is rapid, since the 30 minute test period used was quite adequate to show a response. This contrasts with the finding that when enterotoxin from *Vibrio cholerae* acts on the rabbit ileal loop there is a "latent period" of about 4 hr. (Leich, Iwert and Burrows, 1966). On the other hand the rapid action of *Vibrio cholerae* entero-

TABLE 1

EFFECT OF ENTEROTOXIN ON POTASSIUM AND GLUCOSE ABSORPTION

Figures represent the means of at least five observations ± SE. Positive signs indicate net absorption, negative signs indicate net secretion. The asterisk (*) shows a significant difference between the corresponding enterotoxin and control results ($p < 0.05$. Student's t-test)

		Loop E	*Loop F*	*Loop G*	*Loop H*	*Loop A*	*Loop B*	*Loop I*	*Loop J*
Potassium (μeq/30 min.)	Control	−54±38	−30±16*	−16±12	−36±3	−226±30	−141±34*	−71±8	−103±13
	Toxin	−71±15	−73±16	−28±6	−48±4	−229±28	−234±34	−86±8	−111±12
Glucose (mg./30 min.)	Control	+152±14	+78±10	+27±5	+62±11	+24±4	+27±1	+26±6	+25±4
	Toxin	+145±17	+76±10	+22±4	+48±12	+19±2	+29±4	+13±6	+31±1

TABLE 2

EFFECT OF PHENOL-WATER EXTRACTED ENDOTOXIN (5MG.) ON NET ABSORPTION OF FLUID, SODIUM POTASSIUM, GLUCOSE AND BICARBONATE IN TWO LOOPS

Figures represent the means of 5 observations ±S.E. Positive signs indicate net absorption, negative signs indicate net secretion

Loop		*Fluid absorption ml. per 30 mins.*	*Sodium absorption μ eq per 30 min.*	*Potassium absorption μ eq per 30 min.*	*Glucose absorption mg./30 min.*	*Bicarbonate absorption μ eq per 30 min.*
E	Endotoxin	−3·8 ± 1·0	−550 ± 150	−53 ± 6	+85 ± 3	−66 ± 9
	Control	−4·6 ± 0·8	−580 ± 110	−53 ± 6	+72 ± 4	−14 ± 89
F	Endotoxin	−2·9 ± 0·2	−350 ± 60	−33 ± 20	+58 ± 4	+25 ± 40
	Control	−2·7 ± 0·6	−360 ± 70	−34 ± 90	+45 ± 12	−20 ± 80

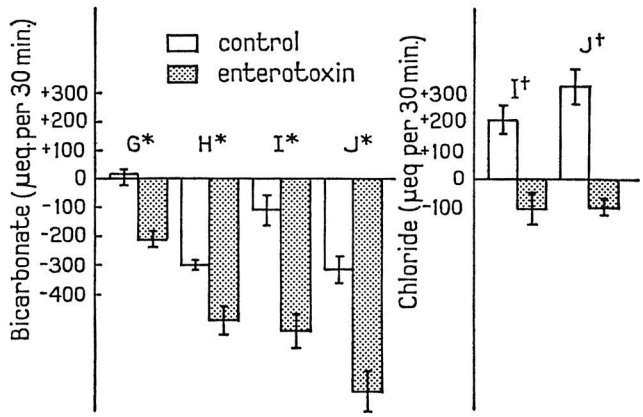

Fig. 2. The effect of enterotoxin on bicarbonate absorption in four loops (G, H, I & J) and on chloride absorption in two loops (I & J). Each column represents the mean of six observations. Positive signs indicate net absorption, negative signs indicate net secretion. Vertical bars represent S.E. Differences are significant in each case, (Student's t-test $p < 0{\cdot}05$ [*] or $p < 0{\cdot}01$ [†]).

toxin on dog small intestine (Carpenter and Greenhough, 1968). is similar to that of *E. coli* enterotoxin.

The effect on fluid, sodium, bicarbonate and chloride movement shows that enterotoxin causes a significant shift towards net secretion, although the effect on potassium movement was inconsistent. These results, excepting potassium, resemble those of *Vibrio cholerae* enterotoxin in the rabbit (Norris, Curran and Schultz, 1969) and in the dog (Swallow, Code and Freter, 1968). Nevertheless,

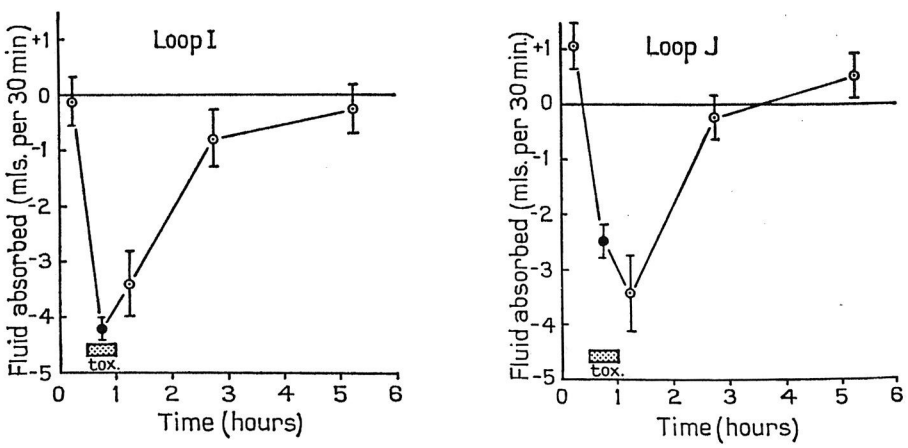

Fig. 3(a) and (b). Absorption of fluid in loops I & J from test solution II (open circles) before and after a 30 minute exposure to enterotoxin (closed circle). Each point represents the mean of five observations. Vertical bars represent S.E. Positive signs indicate net absorption, negative signs indicate net secretion.

the potassium shift caused by *V. cholerae* enterotoxin was small in comparison with the fluid and other electrolyte changes, so that this too is not entirely inconsistent with the present findings. The lack of any effect of *E. coli* enterotoxin on glucose absorption is again similar to the results obtained by other workers using *Vibrio cholerae* enterotoxin in the dog (Carpenter, Sack, Feeley and Steenberg, 1968) and in the rabbit (Serebro, Bayless. Iber and McGonagle, 1968). This may indicate that neither agent has a general toxic effect on mucosal cells, or alternatively that the reserve capacity for glucose absorption is considerable.

The effect of *E. coli* enterotoxin was similar in loops of either upper or lower intestine. This differs from the reports of Smith and Halls (1967a, b) who found that ligated loops in the upper intestine were more sensitive to dilation than the lower intestine, and that the last 3 m., the site of the ileal loops in the present experiments, were unreactive. This difference may be explained by differences in the absorptive behaviour of upper and lower intestine, since 3 out of 4 of the upper loops in the present experiments showed net secretion of fluid, while 3 of the 4 lower loops showed net absorption of fluid. Although these results are not statistically significant, it may be that calf intestine behaves in a similar manner to that of the dog (Carpenter *et al.*, 1968) in that the absorptive capacity of the jejunum is less than that of the ileum. Support for this conjecture is given by the sodium absorption figures from the control solution, which are significantly greater in the ileum than in the jejunum ($P < 0{\cdot}05$). In this case the ligated loops in the posterior part of the small intestine might be expected to show less dilation since their greater absorptive capacity would have to be overcome before dilation could occur.

The enterotoxin preparation used in these experiments was relatively crude and probably contained considerable amounts of culture medium material, much of which would be osmotically active. This could not account for the results obtained, however, since the osmolality of enterotoxic and control solutions did not differ significantly. A further contaminant was possibly non-specific endotoxin (bacterial lipopolysaccharide) which has been shown to be released into culture media under certain conditions (Guckian and Perry, 1966; Marsh and Crutchley, 1967). However, a comparison of the mouse lethality of a concentrated solution of acetone extract of culture fluid with that of phenol-water extracted endotoxin showed that the standard 30 ml. quantity of culture fluid probably contained less than 2 mg. of endotoxin. It was further shown that 5 mg. of endotoxin had no effect on absorption of any of the parameter studied, so it seems unlikely that the enterotoxic effects described can be attributed to endotoxin. This agrees with ligated loop studies which have shown that endotoxin has no dilating ability (Smith and Halls, 1967b; Truszczynski and Pilasek, 1969).

Since the net transfer of fluid and electrolyte in the small intestine is probably small in comparison with either unidirectional flow (Shields, 1964), a change in either insorption or exorption (Code, 1960) will be sufficient to cause a marked effect on net absorption or secretion. The present results may be explained by an increase in exorption, a decrease in insorption, or both, but do not allow a further distinction to be made. The lack of effect on glucose absorption, together with the rapidity in onset of the activity might suggest an action at a superficial level.

The rapid recovery also indicates a reversible effect which probably does not necessitate cell renewal for functional recovery.

These results show that, despite the differences in heat stability and antigenicity indicated by Smith and Halls (1967b), the effect of heat-stable *E. coli* enterotoxin on calf intestine has several features in common with the effect of *Vibrio cholerae* enterotoxin on dog intestine. This is paralleled by the antigenic similarity between heat labile *E. coli* enterotoxin and *V. cholerae* enterotoxin described by Gyles and Barnum (1969), and suggests that a similar mechanism may be involved in the intestinal effects of *E. coli* and *V. cholerae* enterotoxins.

SUMMARY

Thiry-Vella loops in calves were used to show effects produced by *Escherichia coli* enterotoxin on net fluid, glucose and electrolyte transport. A significant shift towards secretion of fluid, sodium, bicarbonate and chloride was shown in all cases where these were examined. The effect on potassium transfer was significant in only two of eight loops examined. Glucose absorption was unaffected.

The action was demonstrable within 30 minutes and could not be accounted for by endotoxin activity or osmotic effects. Recovery of two loops after 30 minutes exposure to enterotoxin was virtually complete within 5 hours.

The effects produced are compared with those caused by *Vibrio cholerae* enterotoxin in other species.

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Some effects of *Escherichia coli* enterotoxin on fluid and electrolyte transfer in calf small intestine

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Certain enteropathogenic strains of *Escherichia coli* produce an enterotoxin which causes dilatation of ligated loops of intestine (Smith & Halls, 1967).

To surmount some of the shortcomings of the ligated loop technique, Thiry-Vella loops were prepared in calves, and the effect of enterotoxin on transfer of fluid, glucose and electrolytes was observed.

Enterotoxin was prepared as described by Smith & Halls. Volumes (30 ml) of culture filtrate were precipitated with 8 volumes of acetone, and the resulting precipitate was redissolved in 30 ml of an electrolyte solution containing polyethylene glycol 4000 as a marker. This was the standard amount used in the present experiments. A control solution contained equivalent amounts of extract from uninoculated culture medium. The solutions containing extract were approximately isotonic.

The net absorption of fluid, sodium, potassium, bicarbonate, chloride and glucose was observed during a control period, and again during an immediately subsequent period in the presence of enterotoxin. Control experiments showed that absorption during such consecutive periods was the same in the absence of enterotoxin.

In each of eight loops examined, the presence of enterotoxin caused net secretion of fluid and sodium ($P<0{\cdot}05$). Loops which absorbed during the control period began to secrete, while those which secreted during the control period showed an increased secretion. Similarly in four loops examined for net chloride and bicarbonate transport, the presence of enterotoxin caused a shift towards secretion in each case ($P<0{\cdot}05$). Potassium absorption was significantly affected in only two of eight loops, while glucose absorption was unaffected in all of eight loops examined.

The effect on net sodium and fluid absorption could have resulted from increased secretion, decreased absorption or both. In order to define the effect more closely, ^{22}Na and deuterium oxide were used as isotopic labels to determine the unidirectional fluxes of sodium and fluid during 10 min periods following exposure to control and enterotoxin solutions. The flux from the lumen was termed insorption, and the flux towards the lumen exorption (Code, 1960).

In the first of two loops in which the unidirectional fluxes were examined, it was found that the presence of enterotoxin caused increased exorption of sodium ($P<0{\cdot}05$). The insorption of sodium was not significantly changed. In the second loop, however, the sodium insorption was decreased ($P<0{\cdot}05$) while the exorption was increased ($P<0{\cdot}05$).

In the case of fluid transfer, in the first loop neither the small decrease in insorption nor the small increase in exorption was itself significant, despite a net shift towards secretion ($P<0{\cdot}05$). In the second loop, however, there was an increased exorption of fluid ($P<0{\cdot}05$) although the insorption was again not significantly altered.

Experiments on four loops in the presence of enterotoxin confirmed that either sodium insorption or exorption could be altered, but the effect on fluid movement in all four was to increase significantly exorption while leaving insorption unchanged.

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DEPRESSED LACTASE ACTIVITY IN THE INTESTINAL MUCOUS MEMBRANE OF CALVES AFTER NEONATAL DIARRHOEA

BY

R. J. BYWATER and W. J. PENHALE

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Depressed Lactase Activity in the Intestinal Mucous Membrane of Calves after Neonatal Diarrhoea

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SUMMARY. Calves which died after scouring had a lower level of lactase activity in the upper intestine than healthy calves. It is suggested that this may be associated with the pathogenesis of the condition.

THE PATHOGENESIS OF neonatal diarrhoea (scour) in calves, in particular the enteric form of the disease (Gay, 1965), is poorly understood. Blaxter & Wood (1953) studied the composition of faeces from scouring and healthy calves and suggested that the primary dysfunction lay high in the alimentary tract, so allowing undigested food to be fermented by bacteria lower down the intestine. Smith (1962) reached a similar conclusion, suggesting that an enzyme deficiency (undefined) may account for the changes he observed, while Radotstits (1965), noted a flattened appearance of intestinal epithelial cells from scouring calves, and suggested that these may be impaired in their ability to digest and absorb nutrients.

Lactose, the main source of carbohydrate in the diet of the newborn calf, is hydrolysed by β-galactosidase (lactase) in the small intestine. The lactase is confined to the outer cell layer of the mucous membrane (Millar & Crane, 1961), and hydrolysis appears to be an intracellular process.

Lactase deficiency has been described in man (Davidson, 1967) and may be primary (congenital), or secondary to damage to the mucous membrane. One cause of such damage in human babies appears to be enteritis (Haworth & Ford 1960; Careddu *et al.* 1963; Sunshine & Kretchmer, 1964). In view of these findings, it seemed possible that calves with neonatal diarrhoea may have a lactase deficiency, either as a primary condition or secondary to mucosal damage. The present experiments were carried out in order to test this hypothesis.

MATERIALS AND METHODS

Intestine from 10 calves was obtained from the

abattoir. The calves were Friesian or Ayrshire bull calves, and the average age was 6 days. The small intestine was removed immediately after the animals had been killed, and was carried to the laboratory in iced saline. On arrival, the intestine was cut from its mesentery along its length, which was measured approximately. Segments of intestine were then removed from points at 5%, 25%, 50%, 75% and 98% of the distance from the pylorus to the ileo-caecal valve. The segments were opened, washed with saline, and the excess saline removed with filter paper. They were then sealed between pieces of Parafilm*, and stored at −30°C. Under these conditions, no loss of activity occurred over a period of at least 1 month.

Samples of intestine from 10 animals which had died after diarrhoea were taken in a similar manner. The average age of these animals was 7 days and all had scoured for at least 3 days before death. The average time elapsing between death and removal of specimens was 6 hr., while the longest was 10 hr. The effect of this delay on lactase activity was tested by placing ligated lengths of normal fresh intestine in a thermos flask containing saline at 38°C. and removing adjacent samples at 3-hour intervals up to 12 hr. In these circumstances the loss of activity was 2·5% per hr. (± 0·46 S.E.) so a corresponding adjustment was made to the results from animals dead for longer than 15 min. assuming that a similar loss occurred in the dead animal.

Samples for assay were thawed, and a sample of mucosa was removed by scraping with the edge of a microscope slide. The wet weight of mucosa was 80-100 mg. This was then washed into an all-glass homogeniser with 2 ml. of ice-cold saline. The sample was homogenised by 25 complete strokes of the plunger. Samples of the homogenate were removed and diluted 10 times for lactase and protein assay.

Lactase activity in the diluted homogenate was measured as described by Dahlqvist (1964) and was expressed as μMoles of lactose hydrolysed per mg. of protein per hour. Optimal substrate pH was 5·65.

Protein was measured using the method of Lowry *et al.* (1951).

RESULTS

Lactase activities in the 2 groups are shown in Fig. 1. Vertical bars represent S.E. of the mean. There are significant differences (Students t-test) between the activities of the two groups at 5% ($P<0.05$), 25% ($P<0.01$) and 50% ($P<0.001$) of the distance between pylorus and ileo-caecal valve. The differences at 75% and 98% do not reach significance ($P<0.05$).

In healthy animals the highest activity appears to lie in the proximal jejunum at the 25% level; although the rise between 5% and 25% is not statistically significant. The fall between 50% and 75% is significant ($P<0.01$).

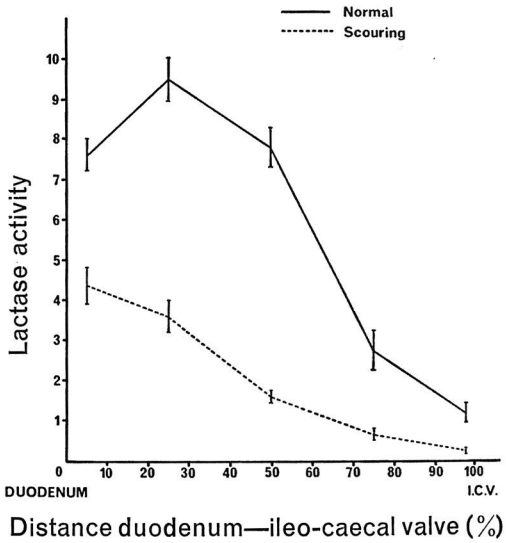

FIG. 1. Lactase activity (μM lactose hydrolysed/mg. protein/hr.) in intestinal mucous membrane from normal and scouring calves. Each point represents the mean of observations on material from 10 animals. Vertical bars represent S.E. of the mean.

DISCUSSION

These results show that the lactase activity of the upper small intestine is significantly reduced in animals that have died after diarrhoea. It is not possible to say whether the observed depression is primary or secondary, although it may be that some calves with a rather low level at birth are particularly susceptible to further lowering following mucosal damage.

The significance of the depression depends on the reserve capacity of lactase activity, and there is some circumstantial evidence that this may be limited. Blaxter & Wood (1953) showed a progressive decrease in dry-matter content of faeces when the lactose plus glucose in the diet was raised above 100 g. (per day) although the proportion of lactose was not stated. Moreover, overfeeding appears to be a predisposing factor in diarrhoea of calves (Roy, 1959).

* Gallenkamp, London.

If the lactase in the gut is inadequate to cope with the lactose in the diet, then undigested lactose will be fermented by micro-organisms in the lower part of the intestine. This would give rise to fermentative diarrhoea as described by Weijers & Van der Kramer (1963) and would aggravate any existing diarrhoea. This hypothesis is in keeping with the findings of Blaxter & Wood (1953) who showed that scouring animals had a lowered faeces pH, and a raised level of fatty acids typical of an abnormal carbohydrate fermentation.

This finding may provide some basis for the well-established practice of reducing the milk intake of scouring calves (Roy, 1959). It may also explain in part the finding of Tennant *et al.* (1968) that some calves in the later stages of colibacillosis show hypoglycaemia.

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